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Evaluating the contribution of tonoplast and plasma membrane transporters in salinity tissue tolerance in barley

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and to the best of my knowledge contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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August 2015

Dedicated to beloved families and friends

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Abstract

Salinity is one of the major abiotic stresses affecting the world food supply. Salt affected soil has area coverage of 950 million ha which accounts for 10% of the land surface of the globe. Fifty percent of the irrigated land (230 million ha) is affected by salinity costing the world food production $US27 billion per annum. In Australia alone, 67% of the agriculture land is affected by transient salinity costing the Australian economy $AUD 1330 million per annum. In comparison to other strategies, the use of salt tolerant plants is cost effective and sustainable way of controlling salinity.

While numerous attempts have been made to develop salt tolerant varieties over the last few decades, most of the efforts were focused on tackling individual components or genes contributing to overall salt tolerance. As a result, the progress in the field was much slower than expected and we still lack truly salt tolerant varieties in the farmers' field. Salinity tolerance is a multi-faceted physiological trait, and all the beneficial effects of improving a function of one gene/mechanism may be lost or overturned by plethora of other contributing factors. This calls for a need of "pyramiding" several key traits in one ideotype. Before this can be implemented into the practice, the essentiality and a relative contribution of various traits should be quantified, for each particular species. The aim of this PhD study was to identifying the major contributing mechanisms to salt tolerance in barley. The major focus was on the following aspects: (1) essentiality of transcriptional vs post translational factors in mediating plant adaptive responses to salinity; (2) quantifying the relative contribution of osmo- and tissue-tolerance mechanisms in barley; (3) identifying components involved in Na^+ sequestration in vacuole; and (4) revealing the role of H^+-ATPase in vacuolar ion sequestration and overall salinity stress tolerance.

In the first part of this PhD study, the relative contribution of ionic, osmotic and oxidative stresses to the overall salinity tolerance in barley was studied, both at the whole

plant and cellular level. In addition, the gene expression profile of key genes in ionic and oxidative homeostasis (*NHX*, *RBOH*, *SOD*, *AHA* and *GORK*) as a way of comparing the contribution of transcriptional and post translational factors for salinity tolerance was also investigated. The major findings can be summarized to two main points. These are: (i) tissue tolerance is the dominant component in which root K^+ retention and lower sensitivity to stress induced hydroxyl radical production are the main ones; (ii) responses at the post-translational level is more important than at the transcriptional level for understanding the mechanisms for salt tolerance. Overall, for better tissue tolerance, sodium sequestration, K^+ retention and resistance to oxidative stress are important salt tolerance mechanisms. It is concluded that every crop improvement programs for salinity stress tolerance should take into consideration all this components.

In the second part of this PhD study, we showed that unlike a number of reports for salt-sensitive "salt excluder" species (such as Arabidopsis or rice), expressing *AtNHX1* in barley had no beneficial effect on plant performance under saline conditions. *AtNHX1* Arabidopsis tonoplast Na^+/H^+ exchanger was expressed in barley (*Hordeum vulgare* L., cv. Golden Promise) and the plants grown under saline growth condition. The transgenic plants were compared with null segregant for biomass, water content, gas exchange, and Na^+ and K^+ content of the leaf. The transgenic barley plants expressing *AtNHX1* has not shown significant difference from the null segregant for any of the trait at least under our experimental conditions. The lack of phenotype in barley which adapts "salt including" strategy was explained by one or more of the following: (i) low level of activity of vacuolar H^+-PPiase and vacuolar H^+-ATPase causing poor proton gradient; (ii) the lack of controlling passive leak of sodium via Na^+ permeable slow activating and fast activating channels in the vacuole; (iii) insufficient ATP pool to assist the H^+ pumping activity; (iv) the AtNHX1 protein may not be folded properly, inactive or mis-targeted.

In the last part of this PhD study, improvement in salinity stress tolerance was obtained in barley crops by expressing V-ATPase subunit C gene. Most of the reports until now have not shown improvement at the grain yield level and also have not explained the physiological mechanisms behind. Also, no previous attempt to express the V-ATPase subunit C was done in barley. Accordingly, we expressed *AtVHA-C* gene in barley and grown under 300mM NaCl. The barley plants expressing the gene were compared with wild type plants for dry biomass, leaf pigment content, stomatal conductance, grain yield, and leaf Na^+ and K^+ content. The transgenic barley plant expressing *AtVHA-C* have shown smaller reduction in biomass and grain yield compared to wild type plants. The beneficial effect in the *AtVHA-C* expressing plant was due to better maintenance of stomatal conductance resulted from the accumulation of Na^+ and K^+ in the leaf that lead to osmotic adjustment and less reliance on the *de novo* synthesis of organic osmolytes.

To recap, salinity tolerance is a multigenic physiological trait involving various mechanisms that may differ between species. For barley, the dominant mechanism appears to be the tissue tolerance. This includes better K^+ retention in the root; reduced tissue sensitivity to oxidative stress; and more efficient sodium sequestration in the leaf. We have shown that for a better sodium sequestration all the components such as proton pumping for generating proton gradient in the tonoplast membrane, control of the back leak of sodium through Na^+ permeable SV and FV channels, and ensuring properly folded, active and correctly targeted NHX protein to the tonoplast all should be considered and modified as one set, to achieve a positive outcome and improve crop salinity tolerance. Finally, we have also shown the importance of vacuolar H^+-ATPase towards salinity tolerance by the contribution of vacuolar H^+-ATPase for the accumulation of Na^+ and K^+ in the leaf where they contribute towards osmotic adjustment, and hence, conservation of energy otherwise spent for organic osmolyte production.

Publications arising from this thesis

(1) Adem GD., Roy S., Zhou M., Bowman J. and Shabala S. (2014) Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. *BMC Plant Biology 14 (1), 113*. (Chapter 3)

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(3) Adem GD., Roy S. Zhou M., Bowman J. and Shabala S. (2015) Expressing Arabidopsis thaliana V-ATPase Subunit C in barley (*Hordeum vulgare* L.) improves plant performance under saline condition by enabling better osmotic adjustment (submitted to *Plant Biotechnology Journal*). (Chapter 5)

(4) Adem GD., Bose J., Zhou M., and Shabla S. (2015) Targeting vacuolar sodium sequestration in plant breeding for salinity tolerance. In H. Wani and A. Hossain (eds.) Managing salinity tolerance in plants: Molecular and Genomic perspectives (pp. 35-50), Taylor & Francis Catalogue # K23522 ISBN: 978-1-4822-4513-4. (Chapter 2)

List of Abbreviations

AHA- Arabidopsis plasma membrane proton ATPase

AtHKT 1;1- *Arabidopsis thaliana* high affinity K^+ transporter 1;1

AtNHX1- *Arabidopsis thaliana* tonoplast sodium/proton exchanger 1

AtNHX2- *Arabidopsis thaliana* tonoplast sodium/proton exchanger 2

AtNHX3- *Arabidopsis thaliana* tonoplast sodium/proton exchanger 3

AtNHX4- Arabidopsis thaliana tonoplast sodium/proton exchanger 4

AtVHA-C- *Arabidopsis thaliana* vacuolar proton ATPase subunit C

AUD- Australian dollar

AVP- Arabidopsis vacuolar pyrophosphatase

betB- betaine aldehyde dehydrogenase

BSM- Basic salt medium

BYDV- Barley yellow dwarf virus

$CaCl_2$- Calcium chloride

CBL10- Calcineurin binding like 10

CDH/betA- Choline dehydrogenase

CIPK 24- CBL interacting protein kinase 24

CodA- Choline oxidase

DW- Dry weight

FV- Tonoplast fast activating Na+ permeable K+ channel

FW-fresh weight

GAPDH2- Gyceraldehyde-3-phosphate dehydrogenase 2

GhNHX1- *Gossypium hirusutum* tonoplast sodium/proton antiporter1

GORK- Gated outward rectified K^+ channel

Gs- Gas exchange/ stomatal conductance

HAK5- Potassium high affinity uptake transporter 5

HKT- High affinity K^+ transporter

HvHKT 2;1- *Hordeum vulgare* high affinity K^+ transporter 2;1

HvNHX1- *Hordeum vulgare* tonoplast sodium/proton exchanger 1

HvNHX2- *Hordeum vulgare* tonoplast sodium/proton exchanger 2

HVP10- *Hordeum vulgare* Pyrophosphatase 10

Hv-PMHATPase- *Hordeum vulgare* plasma membrane H^+ ATPase

Hv-VRT2- *Hordeum vulgare* vernalization gene 2

KCl- Potassium chloride

KOR- Potassium outward rectified channel

LeHAK 5- *Lycopersicon esculentum* high affinity K^+ uptake transporter 5

LeNHX2- *Lycopersicon ensculentum* tonoplast sodium/proton exchanger 2

LIX- Liquid Ion Exchanger

MATE- Multi drug and toxic extrusion gene

NaCl- Sodium chloride

NaClO- Sodium hypochlorite

NADPH oxidase- Nicotine adenine dinucleotide phosphate oxidase

Nax2- Sodium exclusion locus

NHX- Tonoplast sodium/proton exchanger/antiporter

NHX5- tonopast sodium/proton exchanger 5

NHX6- tonoplast sodium/proton exchanger 6

NSCC- Non selective cation channel

$O^{\cdot}_2$- Superoxide

PCD- Programmed cell death

RBoHF- Respiratory burst oxidase subunit F

ROS- Reactive oxygen species

RT-PCR- Reverse transcriptase polymerase chain reaction

RWC- Relative water content

SbMATE- Sorghum bicolor multi drug and toxic extrusion gene

SOD- Superoxide dismutase

SOS1- Salt overly sensitive 1

SOS2- Salt overly sensitive 2

SOS3- salt overly sensitive 3

SsNHX1- *Suaeda salsa* tonoplast sodium/proton exchanger

SV- Tonoplast slow activating Na^+ permeable K^+ channel

TGN-Trans-golgi network

ThNHX1- *Thellinguella halophile* tonoplast sodium/proton antiporter1

TmHKT1;5-A- *Triticum monococcum* high affinity K^+ transporter 1;5-A

TPC-Two pore potassium channel

USDA- United states department of Agriculture

USD- United States dollar

V-ATPase- Vacuolar proton ATPase

VHA- Vacuolar proton ATPase

VMA- Yeast vacuolar proton ATPase

V-PPiase- Vacuolar proton pyrophosphatase

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Chapter 1 General Introduction

1.1 Salinity's Menace to Agricultural Productivity

The earth is overwhelmed by water containing high levels of salt, which is estimated to be on average 30 g of NaCl per litre of water. This salt has negatively affected global crop production and continues to challenge productivity, especially within irrigated lands (Flowers 2004). In the bygone times, farmers were aware that part of their lands going out of use as the land is salt-affected; however, the population in these times were small and hence, not concerned to use this lands for agricultural production (Flowers 1999). Almost 6% of the total land cover of the world is affected by high salt content, which is estimated to be more than 800 million hectare of land (FAO 2008). 50% of the irrigated agricultural land, estimated to be 230 million ha is salt affected (Ruan *et al.* 2010). It is estimated that 67% of Australia's agricultural land is exposed to transient salinity (Rengasamy 2006). This may cost the country more than AUD 1330 million per year (Rengasamy 2002). On top of the loss of economic revenue due to salinity stress, it is also important to increase food production by 50%-70% by 2050 in order to feed the ever increasing world population which is projected to reach 9.3 billion (Brown *et al.* 2008; Millar *et al.* 2012; Ruan *et al.* 2010). As it will be difficult to feed this population on the current arable land, it necessitates the expansion of food production to marginal lands, including salt affected land. Due to climate change the mean annual rainfall in subtropical regions is expected to decrease in the coming few decades. This will force farmers to opt for low quality saline water for irrigation (Barrett-Lennard *et al.* 2010). The problem can be further exacerbated by the competition of biofuel crops for productive cultivated land (Valentine *et al.* 2012). Urbanization is additional problem that encroach agricultural land dwindling vast area of arable land. In Australia, agricultural land has declined by 10% in a decade (2001-2009) due to urbanization (Millar *et al.* 2012). In the face of the above mentioned constraints, the option for increasing agricultural production would be either

intensification of agriculture or expansion of arable land where it is required to utilize marginal land. Due to the high cost of input to intensify agricultural production which reduces profitability and sustainability, the use of marginal lands would be of paramount importance. One of the plausible management methods to make saline soils useful for agricultural production is to grow salt tolerant crops. This can be achieved by searching for physiological and genetic traits in plants responsible for tolerance and fortifying these traits into a genotype either using conventional breeding or molecular breeding tools. Developing a salt tolerant crop is a vital measure both to keep the agricultural productivity sustainable and reclaiming the environment.

Salinity can be explained as the excess ion of soluble salt of Na^{+}, Cl^{-}, Ca^{+2}, SO_4^{-2}, HCO_3^{-} to the level that it can affect growth and development of plants (Mian 2010). The standard method of measuring salinity is by its electric conductivity (EC). The SI unit for this measuring scale is decisiemens per meter (dS/m). A soil is considered to be saline soil when its EC is greater than 4dS/m (Mian 2010). There are two major types of salinity, namely, primary salinity and secondary salinity. Primary salinity happens when salt is accumulated over a long period of time through natural process such as salt drifted from oceans by wind or water and accumulated on a soil or developed through weathering of rocks. The secondary salinity is anthropogenic (happen by human intervention) like the use of salt rich irrigation water and land clearing (Mian 2010). In sea water, NaCl is the dominant salt and the molar concentration of the major ions in sea water includes Na^{+} approximately 460mM, 50mM for Mg, and 540mM for Cl^{-}. Salinity depends upon the evaporation of a particular area under consideration and/or the precipitation of that given land. If precipitation is high, it will reduce the salt concentration. In desert lands, due to a higher evaporation than precipitation, there is a higher accumulation of salts in the soil. High accumulation of salts also occurs in cultivated lands that get heavy irrigation. This is particularly true where the land is in a dry area that

there will be a high evapo-transpiration as consequence the salt present in the irrigation water accumulates in the soil in due course of time. The repercussion of salinity is a far greater loss of arable land and productivity of most of the important crops as most crops are very sensitive to this environmental stress. Glycophytes is a term used to describe salt sensitive plants and it includes rice (*Oryza sativa*), maize (*Zea mays*), Soybean (*Glycine max*) and beans (*Phaseolus vulgaris*) (Mahajan *et al.* 2005). Although, barley is a glycophyte, it tolerates salinity better than other crops.

1.2 Mechanisms of plant salt tolerance

1.2.1 Osmotic adjustment

Similar to water stress, salinity stress reduces the ability of the plants to take up water which quickly reduces the growth rate. In a short time after salt stress exposure, the growth rate decreases and the decrease is the same for salt sensitive and salt tolerant species, for example, durum wheat (salt sensitive) vs. bread wheat (relatively salt tolerant) or barley (relatively salt tolerant) vs. triticale cultivars (Munns *et al.* 1995). The temporary drop in the growth rate and rapid recovery to new reduced rate is due to rapid and transient changes in plant water relations (Munns 2002). Rapid and instantaneous change in the growth rate after salinity exposure have been recorded in different crops and similar change have been observed when mannitol, KCl and polyethylene glycol (PEG) are used (Chazen *et al.* 1995; Yeo *et al.* 1991), indicating that it is a non-ion specific response (Munns 2002). This concept was demonstrated by a pressurization technique reported by Passioura *et al.*, (2000) where the plant water status was maintained by an external pressure and the growth rate reduction was not observed after exposure to salinity and the growth rate transiently surged when salt was removed. Similarly the rapid reduction of growth rate in roots as a result of salt stress by affecting the water relations of the root cells was observed (Rodriguez *et al.* 1997). Similar response in roots was observed by using KCl and mannitol (Frensch *et al.* 1994, 1995). Both responses are due to

sudden change in water relations (Munns 2002). In long term of salinity exposure, that is within days' time, whether water status, hormonal regulation or supply of photosynthate is the dominant growth control in saline soil is an issue debated till now. In a pressurization experiment, it was possible to show that in 8 days of salt treatment no effect on growth was found due to the pressure applied (Munns 1993; Termaat *et al.* 1985). This shows that hormonal and other signals are responsible for growth control rather than leaf water deficit or ion toxicity (Munns 2002). Crops produce osmolytes to offset the osmotic pressure imbalance due to salinity stress; the process referred as osmotic adjustment (Hasegawa *et al.* 2000; Munns *et al.* 2015). However, in barley crops, the accumulation of glycine betaine and proline is inversely correlated with potassium efflux (an indicator of salt tolerance) and total amino acid was poorly affected by salt treatment in salt tolerant barley crops (Chen *et al.* 2007a). These authors showed that the accumulation of compatible solutes was more pronounced in salt sensitive barley crops than tolerant cultivars showing that compatible solutes does not have a major role in salt tolerance in barley. Crops like barley or salt includers tend to use inorganic ions as cheap source of osmotic adjustment, as these ions are less energy costly (Munns *et al.* 2015).

1.2.2 Sodium exclusion

Exclusion of Na^+ from the shoot is a crucial mechanism of salinity tolerance in glycophyte plants such as wheat (Colmer *et al.* 2005; Munns *et al.* 2008). Sodium exclusion avoids a high Na^+/K^+ ratio in the metabolically active cytosol (Gorham *et al.* 1990), a criteria considered as important for salinity tolerance in plants (Maathuis *et al.* 1999; Shabala *et al.* 2008). Glycophytes have three mechanisms for minimizing Na^+ accumulation in the shoot: (i) minimizing of entry of Na^+ into the root; (ii) maximizing Na^+ efflux from the root; and (iii) restricting Na^+ transfer to the shoot. Nevertheless, the unidirectional influx of Na^+ is thermodynamically passive and poorly controlled (Tester *et al.* 2003). Hence, the main

mechanisms for Na^+ exclusion appears to be active extrusion of Na^+ back to the rhizosphere and the restriction of Na^+ loading into the xylem (Cuin *et al.* 2011). In animals, the presence of Na^+ ATPase facilitates the active transport of Na^+ out of the cell (Garciadeblas *et al.* 2001). In plants, the active extrusion of Na^+ from the cytosol is performed by plasma membrane Na^+/H^+ antiporter as shown by the lack of Na^+/H^+ antiporter activity in the plasma membrane of *sos*1 mutant plants (Qiu *et al.* 2002; Qiu *et al.* 2003). Furthermore, sodium extrusion from the cytosol via plasma membrane was also shown by the presence of activity of Na^+/H^+ antiporter using highly purified SOS1 protein reconstituted into artificial proteoliposomes, thereby excluding interferences in the Na^+/H^+ exchange assay by any other spurious transporter (Quintero *et al.* 2011).

Na^+ extrusion has its own disadvantages. The Na^+ extruded from the plant build up in the rhizosphere where it creates an increasing gradient. This leads to increased energy cost and as well become thermodynamically more difficult to extrude salt (Cuin *et al.* 2011). This continuous "futile cycling' of Na^+ could be a problem for the plant and possibly add up to salt toxicity (Tester *et al.* 2003). Under saline growth conditions halophyte plant *Thellingiella halophila*, restricts unidirectional influx of Na^+ instead of increasing efflux, unlike in Arabidopsis (Wang *et al.* 2006). A Similar response was observed in the halophytinc monocot *Piccinella tenuiflora* when compared with wheat (Wang *et al.* 2009). If Na^+ influx into the root is restricted, the energy cost of Na^+ efflux is reduced. Furthermore, sodium extrusion also results in osmotic stress to plants when salt level builds up in the rhizosphere. The build-up of salt in the reduced apoplastic space- the so called 'Oertli effect or hypothesis' could be another possible drawback of a plant's strategy favouring Na^+ efflux over reduced influx. The total exclusion of Na^+ from the plants may not be beneficial in the long term due to the above mentioned reasons (Cuin *et al.* 2011). If Na^+ is sequestered in the cell vacuole, it could be used as osmoticum to aid cell expansion and also reduces Na^+ accumulation in the

rhizosphere. To summarize, the water potential in the plant has to be decreased that it could in turn reduce the need to synthesize compatible solutes which is energetically expensive (Cuin *et al.* 2011).

1.2.3 Sodium sequestration

The restriction of Na^+ loading into the xylem does not contribute to salinity tolerance in barley. This has been observed by the tolerant varieties having as high Na^+ concentration in the xylem as sensitive varieties. The tolerant varieties at the same time maintain high xylem K^+/Na^+ ratios and efficiently sequester the excess Na^+ in the leaves (Shabala *et al.* 2010). Many authors reported that the ability of plants for salinity tolerance is mainly dependent on the exclusion of Na^+ from the shoot [e.g. (Gorham *et al.* 1990; Munns *et al.* 2008)]. This is possible either by restricting the entry of Na^+ from the root into the xylem (Davenport *et al.* 2005; Gorham *et al.* 1990), maximizing Na^+ retrieval from the xylem (Davenport *et al.* 2007), or sending back Na^+ from the leaf into the phloem (Berthomieu *et al.* 2003). Nevertheless, some species utilize Na^+ as cheap osmoticum for maintaining cell turgor (Shabala *et al.* 2010), assuming that it can be properly sequestered in the cell vacuole by the tonoplast Na^+/H^+ exchanger, NHX (Blumwald 2000). In tolerant barley varieties accumulated Na^+ is sequestered somewhere in the leaf blade.

The other supporting evidence for this explanation is that it was shown to increase the palisade parenchyma of tolerant barley varieties by NaCl treatment. In addition to keeping the cytosol free of excess Na^+, it is also important to compartmentalize Na^+ for osmotic adjustment in the leaf cells (Shabala *et al.* 2010). Nevertheless, the compartmentalization of Na^+ in the cell vacuole needs pyramiding of genes involved in the process as well as considering the regulatory components.

1.2.4 Potassium retention

It has been shown that there is a strong negative correlation between K^+ efflux from root and salt tolerance. K^+ efflux from the mature root zone of 3 days old seedling pre-treated with salt is a reliable screening tool for salinity tolerance in barley (Chen *et al.* 2005). Under salt stress, the K^+/Na^+ ratio drops quickly (Maathuis *et al.* 1999). This is due to both accumulation of Na^+ in the cytosol (Zhu 2000) and higher leakage of K^+ from the cell (Shabala 2000; Shabala *et al.* 2003). The K^+ leakage happens because of the NaCl-induced membrane depolarization (Shabala *et al.* 2003). Hence, it's acceptable to use K^+/Na^+ ratio to be used as screening tool for salinity tolerance (Poustini *et al.* 2004; Shannon 1997). However, K^+/Na^+ ratio referred by many breeders is the tissue (root and shoot) K^+/Na^+ ratio rather than the cytosol. Tissue K^+/Na^+ ratio hides the Na^+ compartmentation in the vacuole showing incorrect information which reduces the predictive value of this trait (Chen *et al.* 2005).

The capacity of plant's cell to retain K^+ is important for plant salt tolerance as the ability to exclude or compartmentalize toxic Na^+ (Shabala 2000; Shabala *et al.* 2003). This concept was first tested on seven barley cultivars contrasting in their salt tolerance and proven to be a reliable screening method (Chen *et al.* 2005). In 70 barley cultivars, K^+ flux from the root in response to NaCl treatment was shown to be inversely correlated with relative grain yield, shoot biomass, plant height, net CO_2 assimilation, survival rate and thousand seed weight in glasshouse grown barley plants (Chen *et al.* 2007c). It was shown that the ability of plants to maintain high K^+/Na^+ ratio either by retention of K^+ or by preventing Na^+ from accumulating in leaves is important for salinity tolerance in barley (Chen *et al.* 2007c). For a polygenic trait such as salt tolerance (Flowers 2004), it is difficult to use a single method for 100% predictive value whatever the sensitivity of that method. It was emphasized that a multilateral approach should be used for such complex physiological trait (Chen *et al.* 2007c) such as including other physiological traits with higher correlation with salinity. Using MIFE technique,

measuring both K^+ and Na^+ fluxes would be a highly cost effective and less labour demanding technique to screen barley plants for salt tolerance once the methodological (Cuin *et al.* 2011) and technical issues with Na^+ flux measurement is settled.

1.2.5 Reactive oxygen species detoxification

Reactive oxygen species (ROS) detoxification is also one of the tissue tolerance mechanisms. It was shown that ROS are generated in salinity stressed plant roots and leaves (Miller *et al.* 2008; Mittler 2002). A causal link between ROS and salinity is also becoming evident (Bose *et al.* 2014a). These linkage of salinity with oxidative stress was supported by previous work on H_2O_2 (Maksimovic *et al.* 2013). An increase in ROS production is effected by NADPH oxidase, a cell wall associated peroxidase that produces superoxide by oxidizing NADPH and electron transfer to oxygen (Sagi *et al.* 2006). Hydroxyl radical induced K^+ efflux is mediated by two transport systems, i.e., non-selective cation channel (NSCC) and depolarization-activated K^+ selective outward rectifying channel (Demidchik *et al.* 2003).

1.3 Research aims

The main salt tolerance mechanisms discussed above include osmotolerance, sodium exclusion, sodium sequestration, potassium retention and detoxification of ROS. Among these, the latter three are usually known as components of the tissue tolerance mechanism. The importance and a relative contribution of tissue tolerance mechanisms in the overall salinity stress tolerance is still the matter of debates; the same is true for osmotolerance and Na^+ exclusion. It is also debated of whether adaptation to salinity occurs at transcriptional or post translational level. Some of these gaps in our knowledge were filled in this work. The following major aims addressed were:

- To study the relative contribution of the osmotolerance, sodium sequestration, potassium retention and ROS detoxification in the overall salinity stress tolerance in barley.

- To understand the role of transcriptional and post-translational factors contributing towards above tolerance components.
- To evaluate the feasibility of expression of Arabidopsis Na^+/H^+antiporter *AtNHX1* as a mean for improving salinity tolerance in barley.
- To develop transgenic barley lines expressing *AtVHA-C* (Vacuolar H^+ATPase subunit C) to improve biomass and grain yield under saline growth condition.
- To elucidate the prevalent mechanisms of salt tolerance in *AtVHA-C* expressing barley lines.

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Chapter 2 Targeting vacuolar sodium sequestration in plant breeding for salinity tolerance (literature review),
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Chapter 3 Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley[2]

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Abstract

Salinity tolerance is a physiologically multi-faceted trait attributed to multiple mechanisms. Three barley (*Hordeum vulgare*) varieties contrasting in their salinity tolerance were used to assess the relative contribution of ionic, osmotic and oxidative stress components towards overall salinity stress tolerance in this species, both at the whole-plant and cellular levels. In addition, transcriptional changes in the gene expression profile were studied for key genes mediating plant ionic and oxidative homeostasis (*NHX*; *RBOH*; *SOD*; *AHA* and *GORK*), to compare a contribution of transcriptional and post-translational factors towards the specific components of salinity tolerance. Our major findings are two-fold. First, plant tissue tolerance was a dominating component that has determined the overall plant responses to salinity, with root K^+ retention ability and reduced sensitivity to stress-induced hydroxyl radical production being the main contributing tolerance mechanisms. Second, it was not possible to infer which cultivars were salinity tolerant based solely on expression profiling of candidate genes at one time point in hydroponics. For the genes studied and the time point selected that

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transcriptional changes in the expression of these specific genes had a small role for barley's adaptive responses to salinity. For better tissue tolerance, sodium sequestration, K^+ retention and resistance to oxidative stress all appeared to be crucial. Because these traits are highly interrelated, it is suggested that a major progress in crop breeding for salinity tolerance can be achieved only if these complementary traits are targeted at the same time. Also, the importance of post translational modifications calls for salinity research focus being shifted from merely gene expression study to their functional assessment *in planta.*

Keywords: stomatal conductance; sodium sequestration; potassium retention; membrane potential; tissue specific responses; H^+-ATPase; reactive oxygen species; cytosolic ion homeostasis

3.1 Background

The world food supply is endangered by salinity exacerbated by global environmental warming. Agricultural production is already seriously affected by increasing salinity, with estimated economic penalties being in excess of $12B (Flowers *et al.* 2010; Qadir *et al.* 2008). Creating salt tolerant crop germplasm is, therefore, becoming an urgent imperative (Munns *et al.* 2012; Shabala 2013).

Salinity tolerance in crops is a physiologically multi-faceted trait and is attributed to multiple mechanisms. The key ones include improved osmotic adjustment; minimising Na^+ uptake by roots and/or increasing Na^+ efflux back to the soil; intracellular Na^+ sequestration; potassium retention in the cytosol; tissue-specific Na^+ sequestration; control of xylem ion loading; excluding Na^+ from the shoot; and oxidative stress tolerance (Munns *et al.* 2008; Shabala *et al.* 2012; Zhu 2003). These numerous mechanisms are usually grouped into three major clusters: (i) osmotolerance; (ii) sodium exclusion mechanisms; and (iii) tissue tolerance mechanisms (Munns *et al.* 2008). Despite the significant progress that has been made in elucidating specific details of each of these mechanisms, the relative contribution of the above

components to overall salinity tolerance remain unclear, prompting numerous attempts to overcome the issue by modifying the expression level or function of specific genes by molecular means (Plett *et al.* 2010b; Roy *et al.* 2013).

Crop osmotolerance has long been attributed to a plant's ability to increase *de novo* synthesis of compatible solutes (Hasegawa *et al.* 2000; Shabala *et al.* 2011b). Accordingly, efforts have been made to increase the expression of compatible solute associated genes, most significantly those that catalyse the production of glycine betaine including betaine aldehyde dehydrogenase, encoded by the gene *betB* (Holmstrom *et al.* 2000; Zhou *et al.* 2008), choline dehydrogenase (CDH) encoded by *betA* (Lilius *et al.* 1996) and choline oxidase *codA* (Hayashi *et al.* 1998). Despite these genes being transformed into a large number of species, the success in providing improved crops to the farmers field via this avenue has been very limited (Flowers 2004; Serraj *et al.* 2002).

Another major component of salinity tolerance often targeted in transgenic plants is Na^+ exclusion by minimising Na^+ uptake by the root. This component comes to play by the *SOS1* (plasma membrane Na^+/H^+ exchanger) along with interacting and phosphorylating proteins *SOS3* and *SOS2* forming a Ca^{2+}-dependent signalling cascade (Shi *et al.* 2003; Wu *et al.* 1996). This Na^+ exclusion process is energised by the plasma membrane H^+-ATPase (Palmgren *et al.* 2011). Over-expressing *SOS1* Na^+ exclusion gene or its homologues has been attempted in several species such as Arabidopsis (Shi *et al.* 2003) or tobacco (Yue *et al.* 2012). However, when Na^+ is extruded to the medium by this mechanism, it further increases the osmotic and ionic imbalance that in turn causes the stress to be aggravated. Therefore, such a strategy can only be used as an interim solution and cannot, by itself, confer long term salinity stress tolerance under field conditions. Other mechanisms contributing to restricted Na^+ accumulation in the shoot include reduced Na^+ loading into the xylem (Munns *et al.* 2008; Tester *et al.* 2003) and increased Na^+ retrieval from the xylem (Davenport *et al.* 2007), with a

recent study by Munns *et al.*, (2012) showing that the presence of *TmHKT1;5-A* significantly reduced leaf Na^+ content and increased durum wheat grain yield by 25% compared to near-isogenic lines lacking a Nax2 locus, expressing this gene. Sodium retrieval from the shoot via its recirculation to the root via phloem is another contributing mechanism (Berthomieu *et al.* 2003), this process is also believed to be mediated by HKT transporters (Garciadeblas *et al.* 2003). However, it has been argued that excluding Na^+ from the xylem may not be a plausible mechanism at all times (Shabala *et al.* 2010), as Na^+ can be used as a cheap osmoticum in species possessing high tissue tolerance mechanisms. Indeed barley over-expressing the *HKT* subfamily 2 gene, *HvHKT2;1*, had higher xylem and leaf Na^+ content in saline-grown plants and was correlated with increased salt tolerance (Mian *et al.* 2011).

The third component of salinity tolerance is a tissue tolerance, e.g. an ability of plant to safely handle large amounts of sodium accumulated in plant tissues, without any detrimental effects to cell metabolism. Such tissue tolerance can be achieved by efficient Na^+ sequestration away from the cytosol (where it can affect the metabolic processes of the cell) into vacuole. This is achieved by operation of the tonoplast Na^+/H^+ exchanger (Apse *et al.* 1999; Blumwald 2000; Gaxiola *et al.* 1999) that is energised by the vacuolar H^+-ATPase (V-ATPase, E.C. 3.6.1.3) and pyrophosphatase (V-PPase, E.C. 3.6.1.1.) (Fukuda *et al.* 2004a). The *Arabidopsis thaliana* Na^+/H^+ antiporter gene (*AtNHX1*) was the first plant *NHX* homolog to be cloned (Gaxiola *et al.* 1999). There are six isoforms of *AtNHX* in Arabidopsis identified with *AtNHX1* and *AtNHX2* having being highly expressed in many tissues, while *AtNHX3* and *AtNHX4* are exclusively expressed in flowers and roots (Aharon *et al.* 2003; Yokoi *et al.* 2002). Recently, evidence has emerged suggesting that *NHX1* proteins may operate as K^+/H^+ exchangers, and that their major role may be not in Na^+ transport but in regulation of a vacuolar K^+ content (Barragan *et al.* 2012; Bassil *et al.* 2011b).

Vacuolar Na^+ sequestration is important but not the only mechanism contributing to tissue tolerance. The ability to retain K^+ in cells has recently emerged as an important component of this trait, both in root (Chen *et al.* 2005; Chen *et al.* 2007b; Chen *et al.* 2008; Chen *et al.* 2007d) and leaf (Wu *et al.* 2013) tissues. Strong correlation between K^+ retention ability and plant overall salinity tolerance has been shown in some species (Chen *et al.* 2008; Chen *et al.* 2007d; Smethurst *et al.* 2008) and attributed to the importance of high cytolosolic K^+ to suppress activity of caspase-like proteolytic and endonucleolytic enzymes triggering programmed cell death in salt-affected cells (Demidchik *et al.* 2010; Shabala *et al.* 2007). High cytosolic K^+ is also required to maintain metabolic processes such as protein synthesis by enabling tRNA binding to ribosomes (Wyn Jones *et al.* 1979) Also contributing to plant tissue tolerance is reactive oxygen species (ROS) detoxification. It has been shown that significant amounts of ROS are generated in salt-affected plant tissues in both roots and leaves (Miller *et al.* 2008; Mittler 2002), and the causal link between salinity and oxidative stress signalling and reactive species detoxification is becoming evident (Bose *et al.* 2014a). At the same time, attempts to link plants salinity tolerance with the level of antioxidant activity in their tissues appear to be problematic, with reports being highly controversial and ranging from positive to either negative or no correlations with salinity stress tolerance (Bose *et al.* 2014a; Maksimovic *et al.* 2013).

To the best of our knowledge, only one comprehensive attempt to separate the relative contribution of each of above three major components of salt stress was reported in the literature. Using sophisticated whole-plant imaging facilities to determine the area of healthy leaf and the area of senescing leaf in several einkorn wheat accessions, Rajendran *et al.*, (2009) has reported that the most tolerant genotype (judged by relative growth rate under saline conditions) was the *Triticum monococcum* accession, AUS 18755-4 which was not the best performing in any of the above three major mechanisms contributing to salinity tolerance,

namely Na^+ exclusion, osmotic tolerance, and tissue tolerance, compared to the other eleven accessions studied. On the contrary, this variety had an excellent osmotic tolerance (indexed as 0.95 out of 1) and good tissue tolerance but had rather poor ability to exclude Na^+ (indexed as only 0.17). However, given indirect methods of assessing (e.g. whole-plant phenotyping) and the fact that this work has been conducted on salt-sensitive species (wheat), it remains to be answered to what extent these conclusions can be extrapolated to other species.

Barley is one of the most important cereal crops in the world. While being generally classified as relatively salt tolerant (Maas& Grieve 1984), barley germplasm show a great extent of variability in salinity stress tolerance (Chen *et al.* 2007d; Dai *et al.* 2012). We used this opportunity to examine the salt tolerance mechanisms at post-translational level and compared it with changes observed at the transcript level. Unlike to Rajendran *et al.*, (2009), our assay has been conducted not only at the whole plant but also at the cellular level (using the non-invasive microelctrode ion flux measuring (MIFE) technique). We also aimed to compare a contribution of transcriptional and post-translational factors towards the specific components of the overall salinity tolerance. Our results indicate that root K^+ retention ability and increased tolerance to ROS damage were the main contributing tolerance mechanisms. These traits were pronounced at a post translational but not transcriptionally. The overall poor correlation of the change in transcript levels of selected genes in relation to post translational/functional response, demonstrates the importance of post translational modifications *in planta*.

3.2 Methods

3.2.1 Plant materials and growth conditions

3.2.1.1 Glasshouse experiments

Three barley (*Hordium vulgare* L.) cultivars - Numar (salt tolerant), Naso Nijo (salt sensitive) and Golden Promise (intermediate salinity tolerance) – were used in experiments. Seeds were

obtained from the Tasmanian Institute of Agriculture (TIA) and University of Adelaide Waite Barley Breeders. Seeds were planted into 2L plastic pots containing 70% composted pine bark; 20% coarse sand; 10% sphagnum peat; Limil at 1.8 kg/m^3, dolomite at 1.8 kg/m3). The plant nutrient balance was maintained by adding the slow release Osmocote Plus™ fertilizer (at 6 kg/m3), plus ferrous sulphate (at 500 g/m3) (Bonales-Alatorre *et al.* 2013a). Two levels of NaCl (0 and 150 mM) were applied in five replications and eight plants were grown in each 2L pot. The plants were grown from seed under controlled greenhouse conditions (temperature between 19 and 26 °C; day length, 12 h; average humidity ~65%) at the University of Tasmania between March 2012 and April 2012. The plants were irrigated with salt free water until seedling establishment (approx. for one week) and then after, the plants were irrigated with the respective salt treatments for four weeks.

3.2.1.2 Electrophysiological experiments

Seeds were surface sterilized by 1.5% (w/v) NaClO and rinsed well with distilled water several times. The seeds were germinated and grown for 3 days in an aerated hydroponic solution containing 0.5 mM KCl and 0.1 mM $CaCl_2$ in a dark growth chamber at 24 ± 1^0C as described elsewhere (Shabala 2000; Shabala *et al.* 2010). Plants were used for measurements when their roots were 60 to 70 mm long.

3.2.1.3 Hydroponic experiments

Barley plants were grown in 1L plastic pot in 25% strength of modified Hoagland solution (Epstein 1972) for 3 days. To salt stress the plants, 100mM of NaCl was then added to the hydroponics solution, and plants were grown for additional 8 days. Root and shoot tissues were sub-sampled on days 1, 2, 4, and 8 after salt application. Control plants were grown in 25% strength of Hoagland solution for the entire duration of experiment.

3.2.2 Whole-plant agronomical and physiological characteristics

Shoot fresh (FW) and dry (DW) weight were measured, and relative water content calculated as RWC= (FW-DW)/FW. Before harvesting, leaf chlorophyll content was measured as a SPAD index using a Minolta Chlorophyll Meter SPAD-502 (Konica Minolta, Osaka, Japan) on the third true leaf, at a position about one quarter of the length of the leaf from the leaf tip. Stomatal conductance (Gs) was also measured on the same leaf using a Decagon leaf porometer (Decagon Devices Inc., WA, Australia), under constant light conditions (artificial light of 150µmol$m^{-2}s^{-1}$). The number of necrotic leaves was also counted at harvest.

3.2.3 Tissue sap ion content

Na^+ and K^+ content in plant tissues was determined by the freeze-thaw method essentially as described elsewhere (Cuin *et al.* 2009). In brief, roots of hydroponically-grown plants were quickly rinsed in 10mM $CaCl_2$ to remove apoplastic Na^+, blotted dry and then placed into 1.5 mL microfuge tubes and stored at -20°C. Shoot samples were harvested at the same time and also frozen at -20°C. The frozen samples were thawed and the sap squeezed from the tissue using a pointed glass rod. The sap samples were diluted × 100 times with distilled water, and K^+ and Na^+ content of the sap determined using a flame photometer (MODEL PFP7 Flame photometer, JENWAY, Bibby Scientific Ltd, UK).

3.2.4 Non-invasive ion flux measurements

Net fluxes of K^+ and H^+ were measured using non-invasive ion measurement technique, MIFE (University of Tasmania, Hobart, Australia) as described in our previous publications (Shabala 2000; Shabala *et al.* 2010). In brief, borosilicate glass microelectrodes with the tip diameter of 2 – 3 µm were pulled, silanised with tributylchlorosilane (Fluka, Catalogue no. 90796), and then filled with appropriate back-filling solution. Electrode tips were then filled with an appropriate Liquid Ion Exchanger (LIX) (Fluka Catalogue no. 60031 for K^+; 95297 for H^+). Microelectrodes were calibrated in a set of pH and K^+ standards before and after use.

The electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan) and the tips of the electrodes were drawn close to each other and positioned 40µm above the root surface. While measuring, the electrodes were moving between two positions (40 and 80 µm) in a 10 s square-wave manner. The CHART software records the potential difference between these two positions and converted it to electrochemical potential difference considering the Nernst slope value obtained during calibration. These potential difference values were converted into ion flux using MIFEFLUX software utilising cylindrical diffusion geometry (see Newman, 2001). Ion fluxes were measured from excised root segments of 3 to 4 day old seedlings from elongation (~ 2 mm from the tip) and mature (~ 10 mm) root zones. Root segments were placed in 10 mL Perspex measuring chamber filled with basic salt medium (BSM; 0.5 KCl mM, 0.1 $CaCl_2$ mM, pH 5.7 unbuffered) and allowed to equilibrate for ~ 30 minutes. Steady- state ion fluxes were then recorded for 5-10 min, and then the treatment (either 100mM NaCl – for salinity stress; or 0.3mM $CuCl_2$ + 1mM ascorbate – for ROS stress) was administered.

3.2.5 Gene Expression studies

Barley cultivars were grown hydroponically until the third leaf was fully emerged (plants were approximately 15 days). The growth solution was changed every seven days. Plants were treated with 100mM NaCl for 48 hours, their roots and leaves harvested, and snap frozen with liquid nitrogen. Total RNA was extracted following the method of Chomczynski, (1993), using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Ambion's DNA-free (Madison, WI, USA) was used to remove contamination of genomic DNA. To synthesise cDNA, Invitrogen's superscript III Reverse Transcriptase kit with an oligo$(dT)_{20}$ primer was used, following the manufacturer's instructions. Quantitative Real-time PCR was performed as described in (Burton *et al.* 2008) using a RG6000 Rotor-Gene real time thermal cycler (Corbett Research, Sydney) and SYBR® green PCR reagent (Bio-Rad Laboratories,

Gladesville). Primers were designed to determine the expression of a number of key genes involved in Na^+ compartmentation and ROS detoxification and included members of the *NHX*, *AHA*, *RBoHF*, *SOD* and *GORK* family of genes. Primer sequences can be found in Table 3.2. Normalization of the test gene transcript was relative to the control gene (*GAPdH2*).

3.2.6 Statistical Analysis

All the values in this manuscript are presented as mean value ± SE. For mean comparison and statistical significant level paired wise t-test in all possible combination of the treatments was employed using SPSS software version 20 (IBM support portal, USA).

3.3 Results

3.3.1 Whole-plant physiological responses

Salinity stress significantly affected plant growth and biomass production (Fig. 3.1A), resulting in a three- and five-fold reduction in the fresh weight in the tolerant variety Numar and the sensitive cultivar Naso Nijo, respectively (FW; Fig. 3.1B). The cultivar Golden Promise displayed an intermediate salinity tolerance with a 4-fold FW reduction in biomass production under salt stress. All the differences were significant at $P<0.001$.

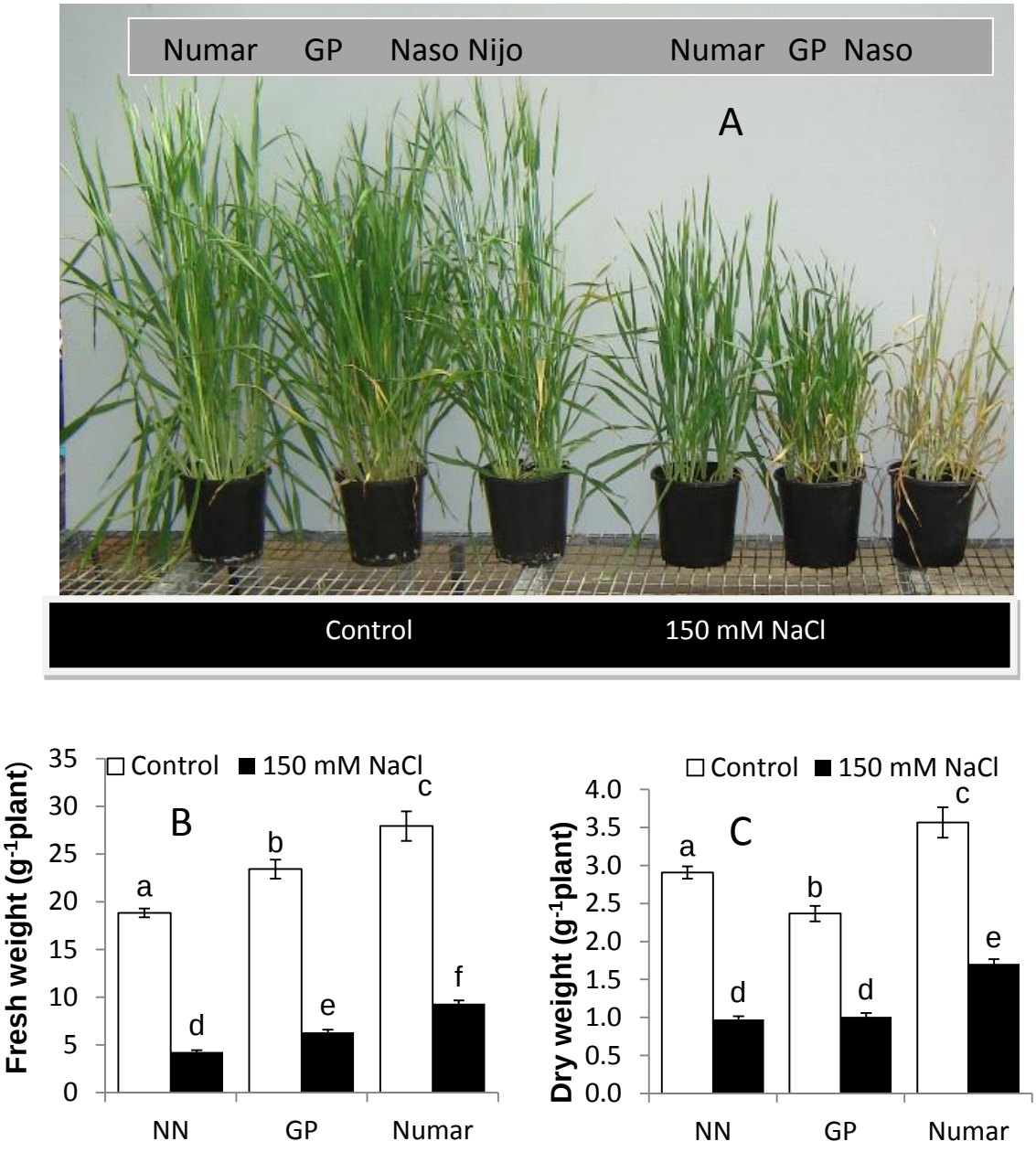

Figure 3.1 Growth responses and agronomical characteristics of three barley cultivars (Numar, Golden Promise, and Naso Nijo) treated with 150 mM NaCl for 4 weeks. A – plant phenotype under control and salt conditions; B – shoot fresh weight; C – shoot dry weight. Open bar - control; closed bar - salt. Mean ± SE (n=30).

Four weeks of salinity stress also resulted in a reductions in leaf chlorophyll content (Fig. 3.2A). This reduction in chlorophyll varied significantly among cultivars, with the salt

sensitive Naso Nijo exhibiting the highest reduction in chlorophyll content (a 4-fold reduction from 29 ± 0.7 to 7.9 ± 1.6 arbitrary units; significant at $P < 0.001$). The salt-tolerant cultivar Numar, however, increased its leaf chlorophyll content by ~ 10% (significant at $P < 0.05$), while the intermediate salt tolerant Golden Promise showed no significant reduction in chlorophyll content. The chlorophyll content of the salt grown Numar leaves was 4-fold greater than that measured in salt stressed Naso Nijo leaves (all the differences are significant at $P < 0.01$: Fig. 3.2A). The salt-sensitive Naso Nijo had twice as much necrotic leaves as Numar and Golden Promise (Fig 3.2B)

Salinity stress also resulted in a significant ($P < 0.01$) decline in stomatal conductance (Gs; Fig 3.2C) and shoot water content (RWC; Fig. 3.2D). The highest decline in Gs was measured in the sensitive cultivar Naso Nijo (a five-fold reduction from 53.2 ± 4.7 to 10.4 ± 2.3 mmol m^{-2} s^{-1}). Surprisingly, the intermediate cultivar Golden Promise outperformed salt tolerant variety Numar (2-fold vs 4.5-fold Gs reduction, respectively; both significant at $P < 0.01$). However, due to initially higher Gs values in control conditions for Numar, the difference in Gs between salt-grown Golden Promise and Numar genotypes was not statistically significant (at $P < 0.05$). As a result, both these varieties retained significantly ($P < 0.01$) more water in the shoot compared with salt-sensitive Naso Nijo (Fig. 3.2D).

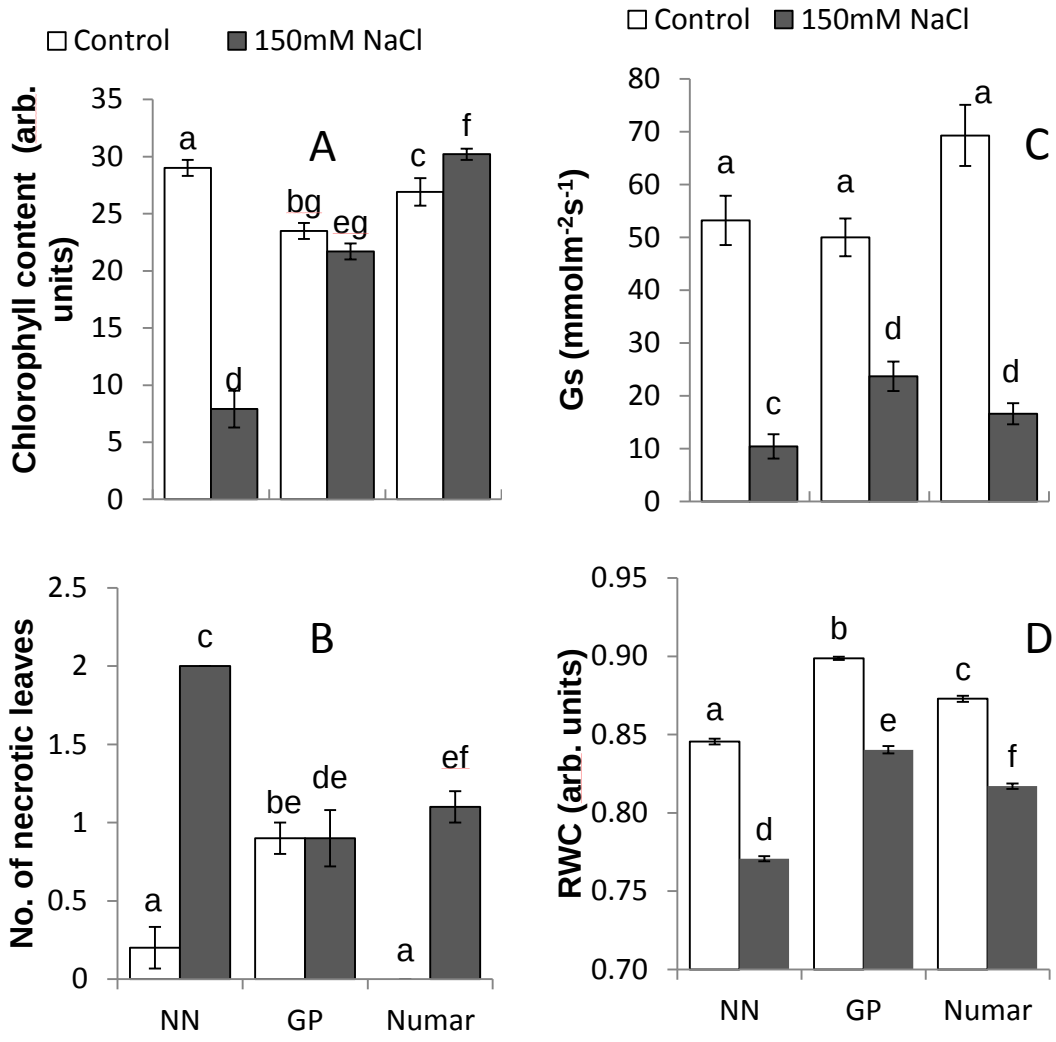

Figure 3.2 Whole-plant physiological characteristics in control- and salt-grown (150 mM NaCl for 4 weeks) plants. A - chlorophyll content (SPAD readings); B - number of necrotic leaves; C – stomatal conductance (Gs); D – relative water content. Open bar - control; closed bar - salt. Mean ± SE (n = 10 and 30 for A-C and D, respectively).

3.3.2 Tissue ionic relations

Hydroponic experiments demonstrated that salt-tolerant Numar accumulated less Na^+ in the root (Fig 3.3A) compared with two other cultivars, throughout the whole salt stress period. At the same time, Numar plants had twice as much Na^+ in the shoot sap compared with other cultivar on day 4 (Fig. 3.3B), however, similar shoot Na^+ were observed between all three cultivars by day 8 (Fig. 3.3B). There was no significant difference in either root or shoot Na^+ accumulation between the three cultivars when grown in the absence of salt (data not shown). Salinity stress resulted in a rapid and progressive decline in root K^+ content in salt-sensitive cultivar Naso Nijo (a 2-fold decrease from 128.4 ± 31 to 62.4 ± 2.7 mM over 8 days of 100 mM NaCl treatment; Fig 3.3C), while over the same time period, root K^+ content did not change significantly in salt-tolerant cultivar Numar. The root K^+ content in the intermediate salt tolerant Golden Promise also declined substantially, but not to the same extent as in Naso Nijo (Fig 3.3C). Interestingly, while root K^+ content dropped sharply in cultivars Naso Nijo and Golden promise, their shoot K^+ content increased approximately 2 fold over the same time period (Fig. 3.3D), suggesting a possible retranslocation of K^+ from root to shoot. This observation was not seen for Numar (Fig 3.3D). The mean values for root K^+ content in controls were 111.2±11.6, 153±4.5 and 146.7±1.9 mM, for Naso Nijo, Golden Promise and Numar, respectively.

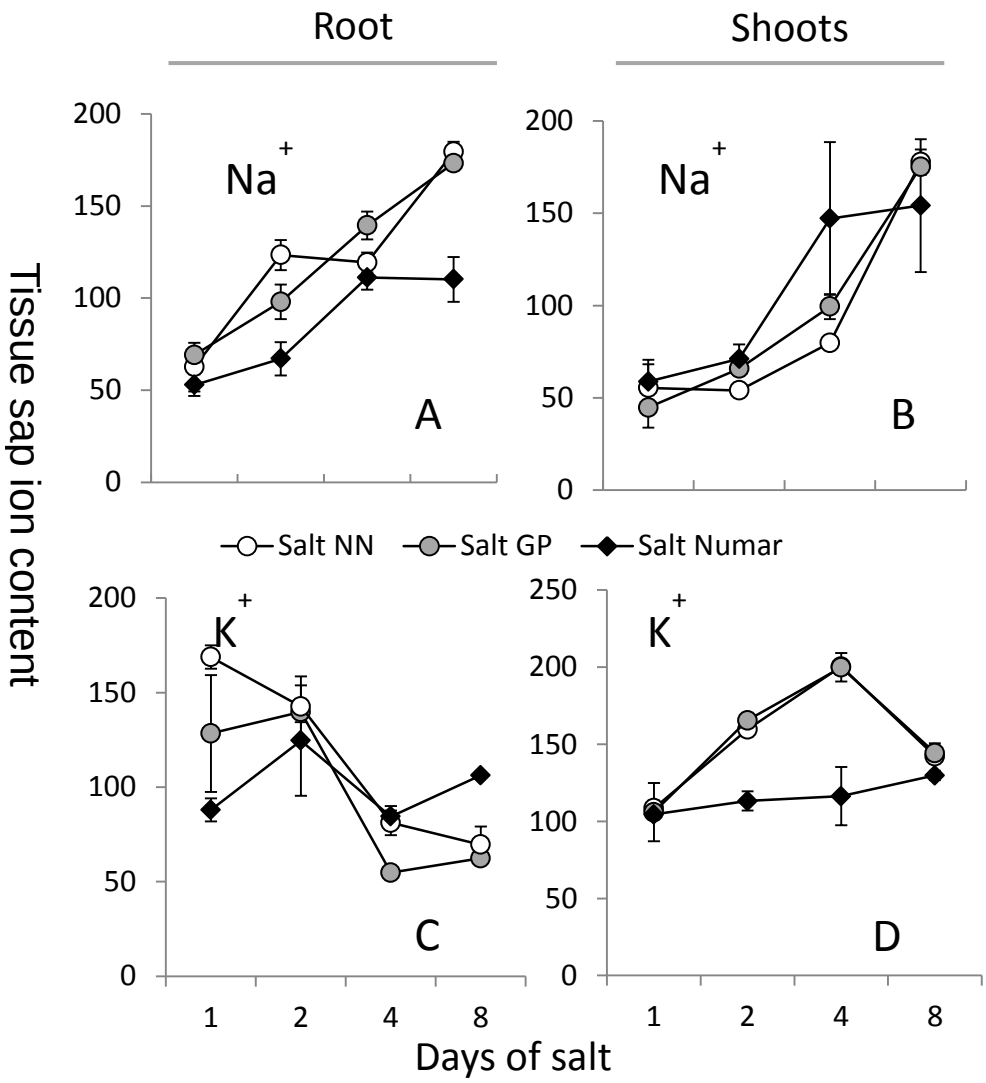

Figure 3.3 Changes in root and shoot sap Na+ and K+ content in three barley cultivars contrasting in their salinity stress tolerance during salt stress progression. Plants were grown hydroponically under control conditions until 3 days old, and then exposed to 100mM NaCl treatment. Mean ± SE (n=24).

3.3.3 Salinity- and hydroxyl radical-induced ion flux kinetics

Similar to our previous reports on barley (Chen *et al.* 2005; Chen *et al.* 2007d; Maksimovic *et al.* 2013), acute NaCl treatment induced massive K^+ efflux from the epidermal cells of the root (Fig. 3.4). Epidermal cells in elongation zone had approximately 5-fold higher K^+ efflux when compared with those in mature zone (Figs 3.4A and B, respectively; significant at $P < 0.01$). Responses of cultivar Golden Promise and Numar were very similar (no difference at $P < 0.05$) while the magnitude of NaCl-induced K^+ leak from the roots of the salt-sensitive Naso Nijo was approximately 4-fold greater than the response seen in the other two cultivars (Fig 3.4; significant at $P < 0.05$).

Salinity treatment has also induced a rapid (within one minute) net H^+ efflux from both the elongation and mature root zones (Fig. 3.5). This activation was strongest for Golden Promise, followed by Numar and then Naso Nijo (Fig. 3.5). The difference in H^+ efflux amongst cultivars was significant at $p<0.01$; and so was the difference between elongation and mature zones (Fig. 3.5A & B). The NaCl-induced H^+ efflux was more pronounced in the elongation zone in comparison to the mature zone.

Consistent with previous observations (Cuin *et al.* 2007; Demidchik *et al.* 2010), addition of hydroxyl radical-generating Cu/ascorbate mix has also triggered massive K^+ efflux from plant roots (Fig. 3.6). Similar to NaCl stress, responses from elongation zone for each cultivar was significantly ($P < 0.05$) stronger compared with mature root zone (Fig. 3.6A and 6B, respectively). ROS induced K^+ efflux was higher in the salinity sensitive cultivar Naso Nijo followed by intermediate Golden Promise variety and then the tolerant cultivar Numar (Fig 3.6B; significant at $P<0.05$).

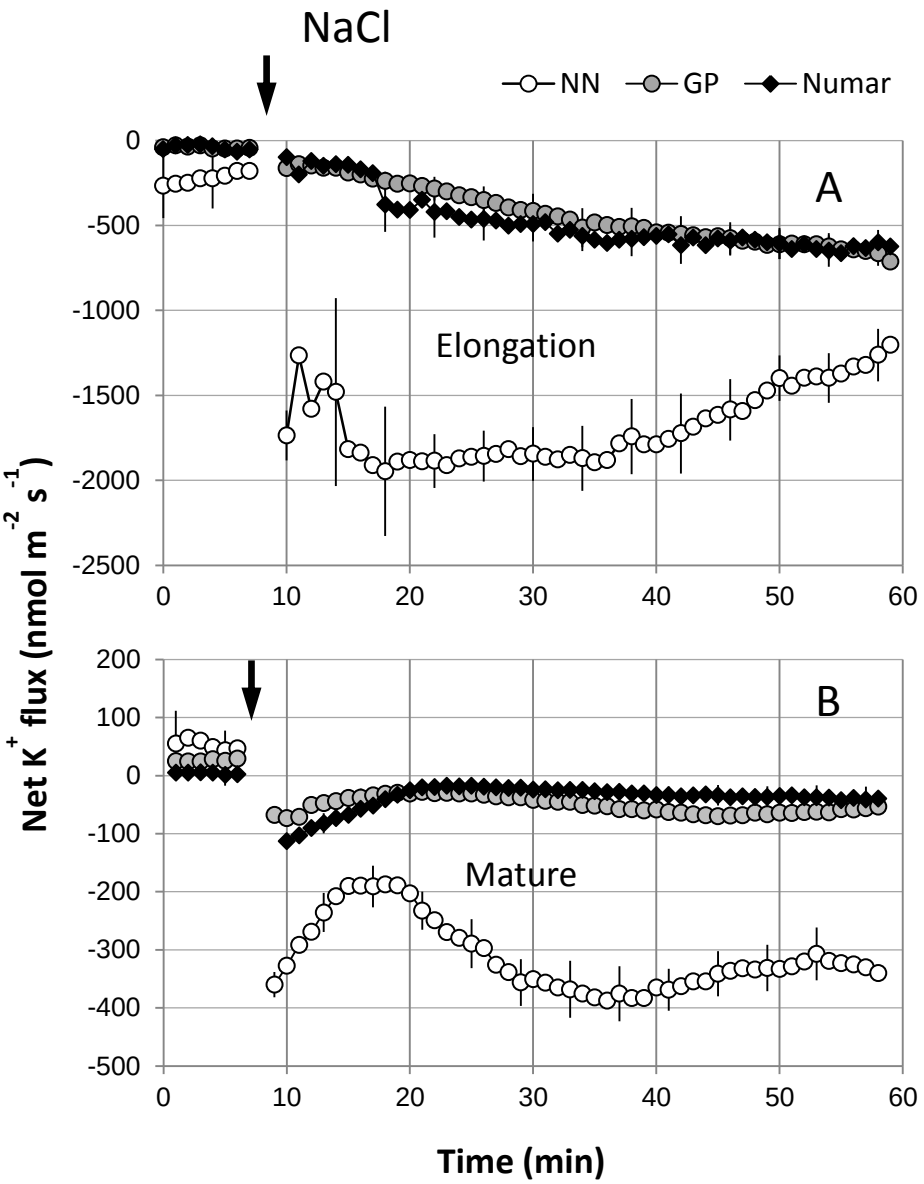

Figure 3.4 Kinetics of NaCl- induced net K^+ fluxes measured form the elongation (A) and mature (B) root zones of three barley cultivars in response to 100mM NaCl treatment. Mean ± SE (n=6-8). The sign convention is "efflux negative".

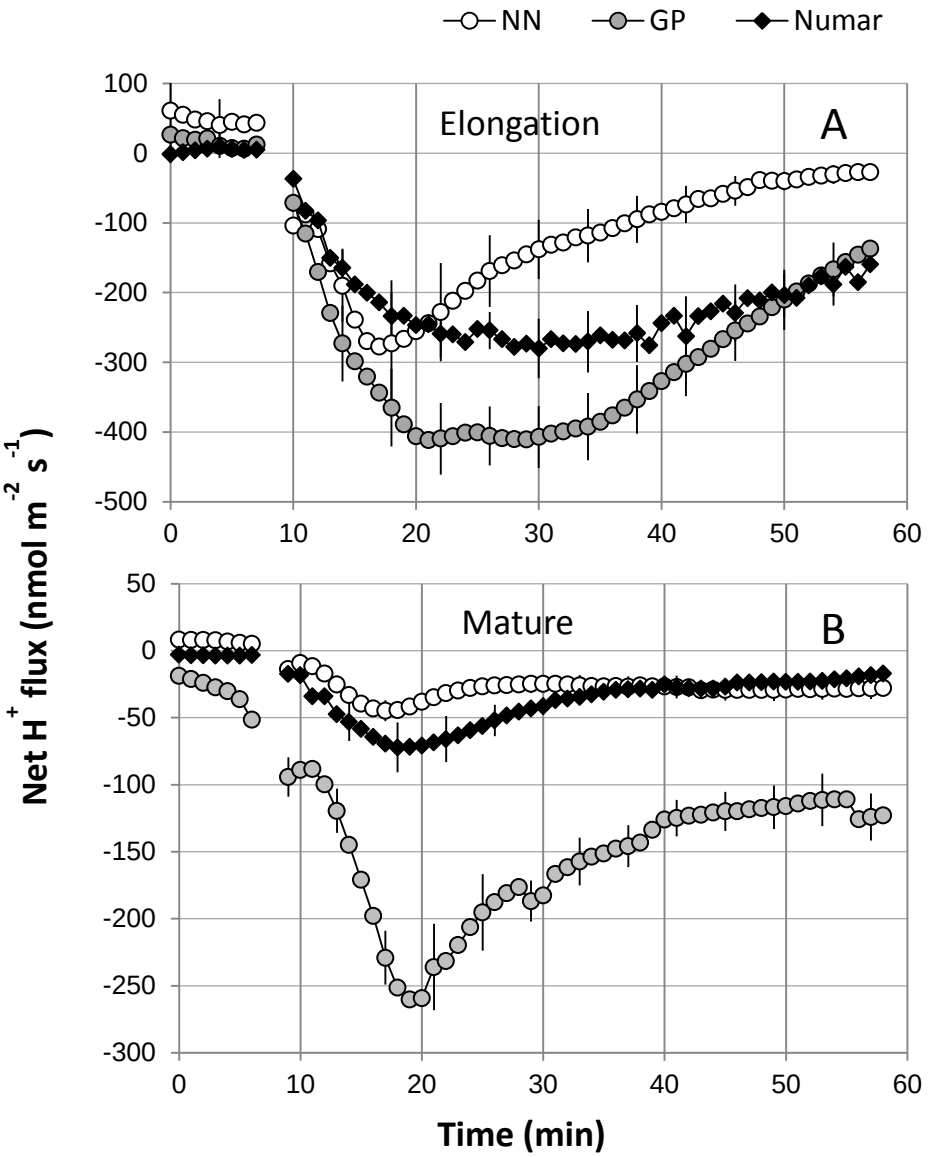

Figure 3.5 Kinetics of NaCl- induced net H^+ fluxes measured from the elongation (A) and mature (B) root zones of three barley cultivars in response to 100mM NaCl treatment. Mean ± SE (n=6-8). The sign convention is "efflux negative".

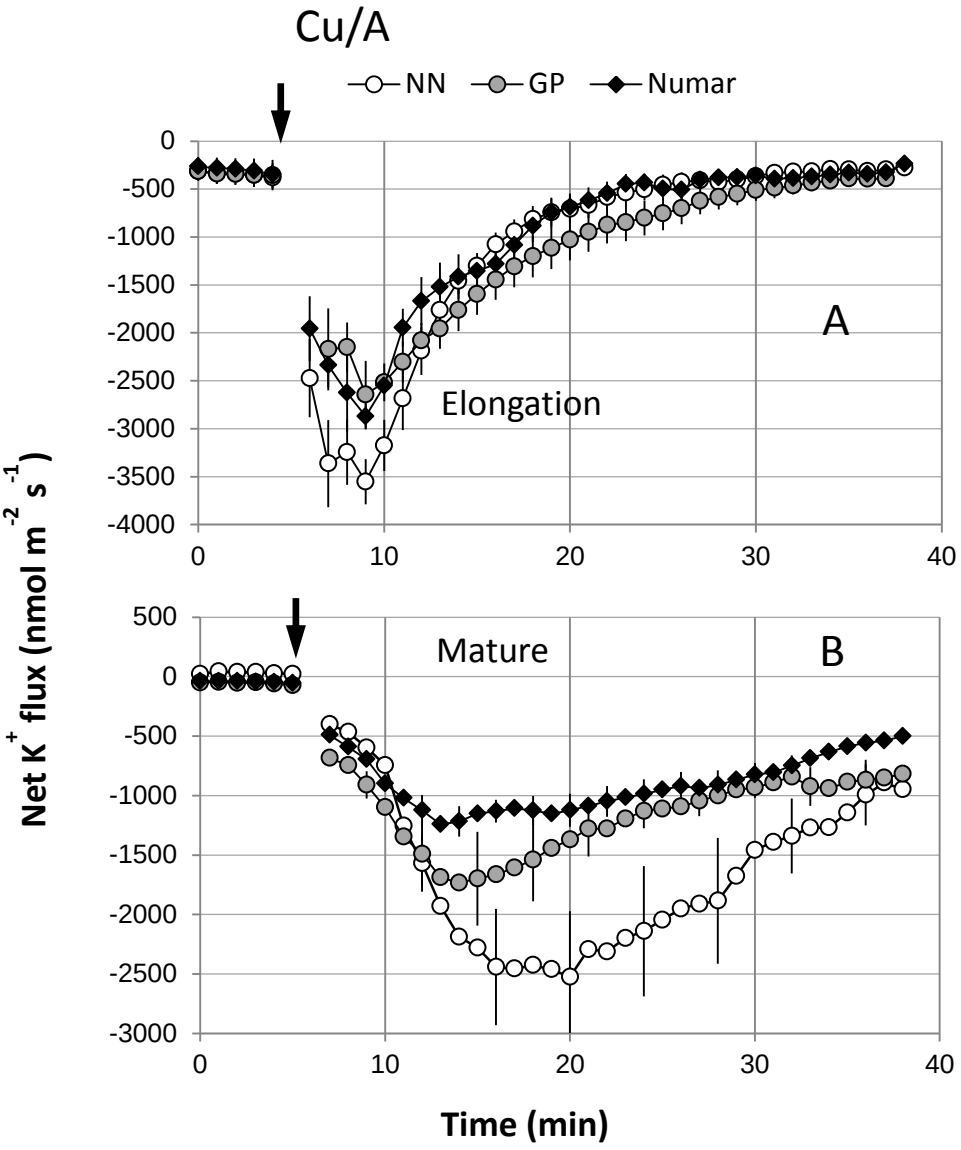

Figure 3.6 Hydroxyl radical-induced changes in net K^+ flux measured from the elongation (A) and mature (B) root zones of three barley cultivars. The hydroxyl radical-generated copper ascorbate mix (0.3 mM $CuCl_2$ and 1 mM Na^+-Ascorbate) was added at the time indicated by an arrow. Mean ± SE (n=6-8). The sign convention is "efflux negative".

3.3.4 Transcriptional changes in the gene expression profile

The expression of a number of key genes involved in Na^+ compartmentation and ROS related were examined in hydroponically-grown plants (Figs 3.7, 3.8; Table 3.1). The expression pattern of *GORK* was also examined, given the essential role cytosolic K^+ retention plays in salinity tolerance in barley (Chen *et al.* 2005; Chen *et al.* 2007d) .with the GORK channel having a key role in this process (Shabala *et al.* 2008).

As shown in Fig. 3.7 and 3.8, there was a large range in the transcriptional response in the eight genes studied to salt application in both leaves and roots. However no clear patterns of gene expression could be observed which could explain the difference in tolerance between the three cultivars. Of three homologues to the *AtNHX* gene, which was originally suggested to encode a protein involved in Na^+ sequestration into the vacuoles, a 2-fold increase in *NHX1* transcript level was measured both in the leaf and root of the salt sensitive NasoNijo (Fig 3.7). No significant changes in *NHX1* expression were measured in the leaf and root of the salt-tolerant Numar plants, while in the intermediate Golden Promise significant *NHX1* upregulation was only observed in the leaves (Fig 3.7). *NHX2* transcript levels went down in both leaves and roots in cultivars Numar and Golden Promise but not in salt-sensitive NasoNijo (Fig. 3.7), while no clear patterns were observed for *NHX3* transcripts.

During salinity treatment there was a relative increase in the expression of the barley homologue *AHA2* in the roots of both Numar and Naso Nijo, while a significant induction was observed only in the leaves of Naso Nijo (Fig. 3.7). No significant ($P < 0.05$) changes in *AHA2* transcript levels were detected in Golden Promise plants.

The relative gene expression of barley homologue of *RBoHF1,* important in encoding for NADPH oxidase was down-regulated significantly ($P<0.01$) by about 2-fold in the leaves of all the cultivars after salt application (Fig. 3.8), however, no clear patterns in the roots were detected. *RBoHF2,* also involved in the production of NADPH oxidase was only slightly

down-regulated after salt application (significant for Numar in leaves and Naso Nijo and Golden Promise in roots; Fig 3.8). The barley homologue of *SOD2* relative gene expression showed small increase in leaves of Golden Promise cultivar but no significant changes for any other varieties in either tissue (Fig. 3.8).

The largest and most striking difference was observed for NaCl-induced changes in the relative transcript abundance of the barley homologue of *GORK* in barley roots, with 4 to 5-fold increase reported for all cultivars (Fig. 3.8; all significant at $P < 0.01$). In leaves, small but significant (at $P < 0.05$) increase in *GORK* transcript level was measured in the salt-sensitive Naso Nijo while the *GORK* transcripts in the leaf of salt-tolerant Numar were reduced (Fig. 3.8).

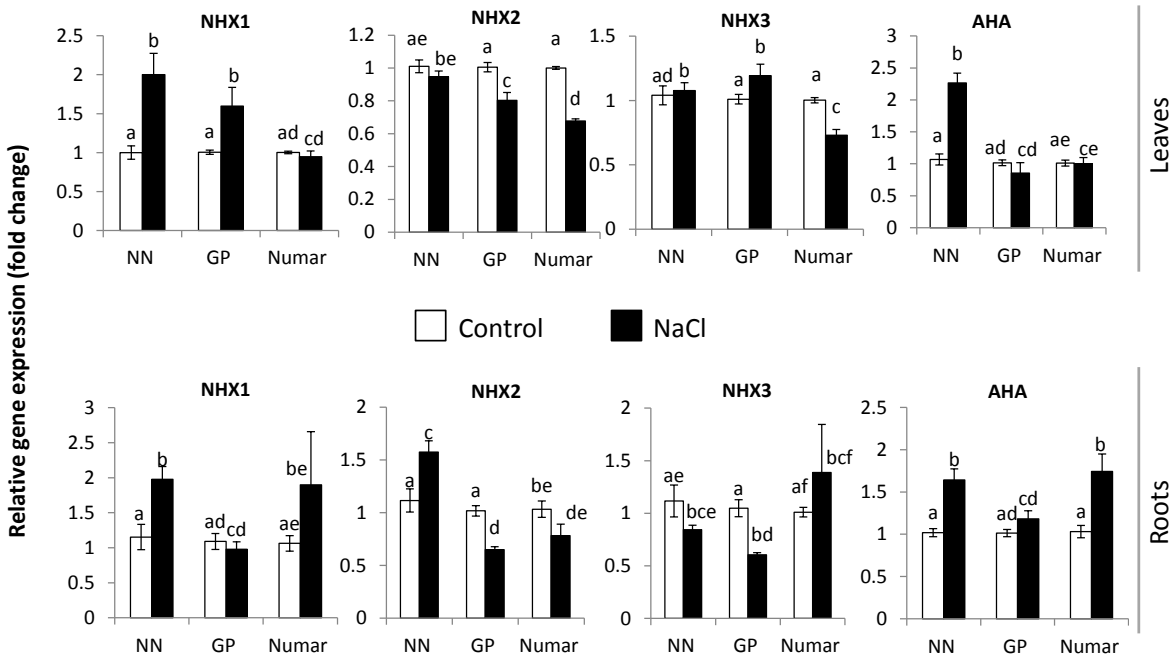

Figure 3.7 Expression of barley *NHX1*, *NHX2*, *NHX3*, and *AHA2* transporter genes in leaf and root tissues after 48 h of 100mM NaCl treatment. Mean ± SE (n=12-15).

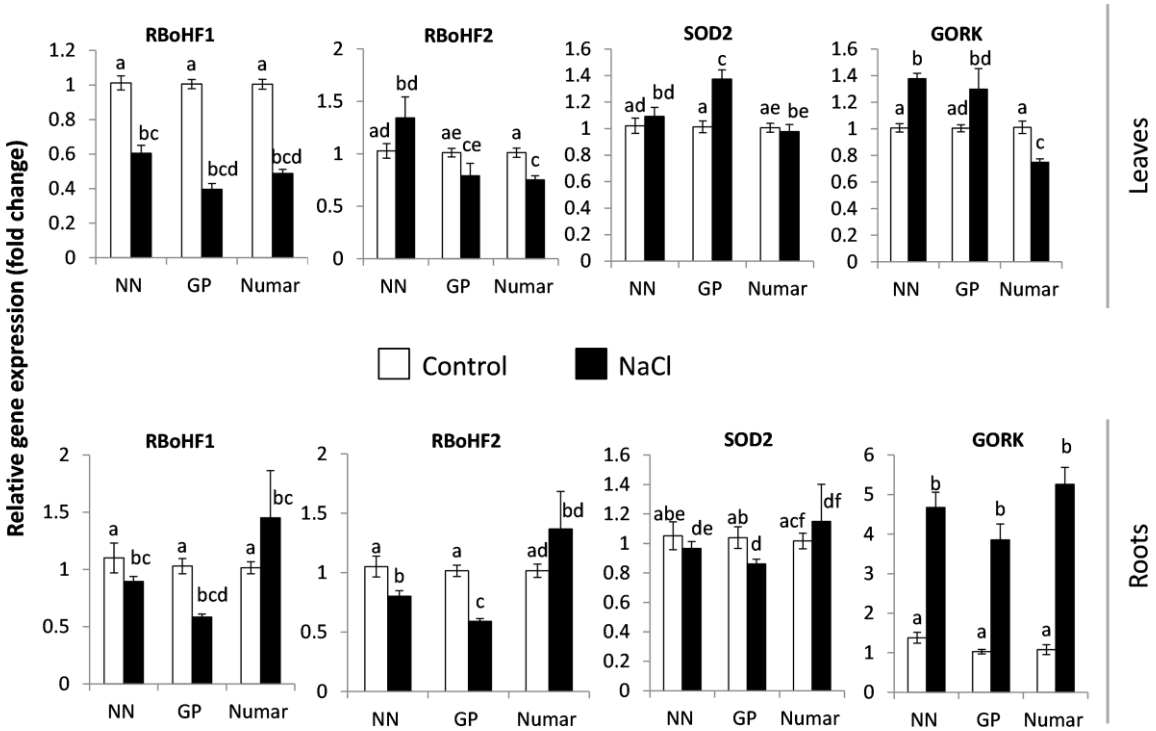

Figure 3.8 Expression of barley *RBoHF1* (Respiratory oxidative burst homologue/NADPH oxidase), *RBoHF2*, *SOD2* and *GORK* genes in leaf and root tissues after 48 h of 100mM NaCl treatment. Mean ±SE (n=12-15)

Table 3.1 The relative gene expression of the gene studied in fold change presented as percentage.

Gene Name	% Fold Change (p-value)			% Fold Change (p-value)		
	Leaf			Root		
	Cultivar			Cultivar		
	NN	GP	Numar	NN	GP	Numar
Hv-NHX1	200	160	-95	171	-89	178
	(0.005)	(0.02)	(NS)	(0.01)	(NS)	(NS)
Hv-NHX2	-94	-80	-68	141	-64	-76
	(NS)	(0.0001)	(0.0001)	(0.03)	(0.0001)	(NS)
Hv-NHX3	104	118	-73	-76	-58	137
	(NS)	(0.01)	(0.0009)	(NS)	(0.0001)	(NS)
Hv-RBoHF1	-60	-39	-49	-81	-57	143
	(0.0001)	(0.0001)	(0.0001)	(NS)	(0.0001)	(NS)
Hv-RBoHF2	131	-78	-74	-76	-58	134
	(NS)	(NS)	(0.0001)	(0.05)	(0.0001)	(NS)
Hv-PMHATPase	212	-84	99	161	117	169
	(0.0001)	(NS)	(NS)	(0.0005)	(NS)	(0.02)
Hv-SOD2	107	136	97	-92	-83	113
	(NS)	(0.0001)	(NS)	(NS)	(0.05)	(NS)
Hv-GORK	137	129	-74	339	375	487
	(0.006)	(NS)	(0.0001)	(0.0001)	(0.0001)	(0.0001)

NS-Non-significant; - sign indicate down-regulation

Table 3.2 The Primers used in the gene expression study and their respective amplicon size.

Gene Name	Forward Primer	Reverse Primer	Amplicon Size (bp)
Hv-NHX1	TGCATATCTACCA-GTGCTTAT	GGTTCAAGACACA-AGTTCAGT	184
Hv-NHX2	GGTTTTCGGCTTG-CTGACTAA	CATTGGGCGCATG-AACTTATC	238
Hv-NHX3	TGAGCCGAACATT-ACTGTGAT	ACGAGCTTACCTT-TCAATACA	127
Hv-RBoHF1	TTACAACATGGAC-CTGCGTCCCTACA	TGCCTTGGTCAGA-CACTCAGCTGCAT	206
Hv-RBoHF2	TATGCGGAGTCCC-GCAGAAAGATG	TGTACTGTACTCC-CCCTGCCTGTGT	212
Hv-PMHATPase	CTTGGTTATCGCC-TTCCTTC	GCCACTCAGCACA-AATCG	184
Hv-SOD2	CTTGAAGGACACC-GACTTGC	CTCAAAAAGCCA-AATGACAGTG	140
Hv-GORK	CCACACGAGGCGA-AGAAG	GAGGAATCCACAG-CATCACC	194
Hv-GAPdH2	GTGAGGCTGGTGC-TGATTACG	TGGTGCAGCTAGC-ATTTGAGAC	Control gene

3.4 Discussion

3.4.1 Transcriptional changes in gene expression play a small role in barley adaptive responses to salinity

Vacuolar sodium sequestration is essential to avoid Na^+ cytotoxicity, and increased activity of *NHX* tonoplast Na^+/H^+ exchangers was shown to enhance salinity tolerance in plants (Apse *et al.* 1999; Blumwald *et al.* 2000). However, in the work presented here a 2-fold increase in *NHX1* transcript level was measured in both leaves and roots of salt-sensitive Naso Nijo (Fig 3.7), while no significant (at $P < 0.05$) changes were measured in salt-tolerant Numar plants in either tissue. Consistent with this, *NHX2* transcript levels went down in both tissues in cultivars Numar and Golden Promise but not in salt-sensitive Naso Nijo (Fig. 3.7). Thus, it appears that changes in transcript levels of the *NHX*s have a little role (and low predictive value) on the salinity stress tolerance in barley in our experiments and changes in expression cannot be used to predict salt tolerance.

Several possible explanations should be considered for these observations. First, as described above, there is recent evidence that the major role of *AtNHX1* may be in K^+ but not Na^+ transport (Bassil *et al.* 2011b), thus the genes studied here may not encode proteins involved in Na^+ compartmentation. Second, the higher NaCl induced expression of *NHX*s in the shoots of the salt-sensitive Naso Nijo could be to compensate for its inability to prevent Na^+ delivery to the shoot (e.g. a need to deal with the consequences of cytosolic Na^+ accumulation). However, this explanation is unlikely as the shoot Na^+ content of the salt tolerant Numar was higher than that of NasoNijo between days 2 and 4 of salt stress (the time when gene expression of the *NHXs* were assessed). Another explanation is that post-translational modifications such as protein folding are much more important that gene expression to have a proper functional response – the proteins are already present at desired concentrations (therefore gene expression is not required), however, need to be activated by

post-translational modifications, a process which allows a plant to respond faster to stress than relying solely on gene expression. It should also be noted that only one time point was used for the expression studies, and expression of genes encoding salt tolerance gene has been shown to fluctuate between days (e.g *HVP10* in (Shavrukov *et al.* 2013)). Lastly, it should also be noted the *NHX1* and *NHX2* genes in barley are homologues of *AtNHX1* and, while having similar nucleotide sequence may not necessarily confer the protein(s) function as AtNHX1 in Arabidopsis.

The above notion that the changes in transcript levels do not always confer plant salinity stress tolerance is further corroborated by study of plant oxidative stress responses. No significant difference in the relative expressions of *RBoHF1* and *RBoHF2* which encode the NADPH oxidase/*Nox* (one of the major sources of ROS production under saline conditions; (Bolwell *et al.* 1997; Kawano 2003)) were found in roots of the salt sensitive NasoNijo and salt tolerant Numar cultivars (Fig. 3.8). Similarly, salt stress did not result in any significant changes in *SOD* expression level in either root or leaf tissues of these varieties (Fig. 3.8). At the same time, NasoNijo roots were twice more sensitive to ROS treatment (Fig. 3.6B).

Other evidence comes from comparing the NaCl-induced net H^+ fluxes measured in barley roots (Fig 3.5) with the changes in the *AHA* (encoding plasma membrane H^+-ATPase) transcript levels (Fig 3.7). H^+-ATPase activity is indispensable for maintaining membrane potential (Palmgren *et al.*, (2011), and intrinsically higher H^+-ATPase activity was shown to correlate with salinity tolerance in barley (Chen *et al.* 2007d). In this study, the Golden Promise cultivar showed consistently higher proton efflux in both elongation and mature zone compared with two other cultivars (Fig. 3.5A, 3.5B). However, the expression of the *AHA* barley homologue was shown to be lower compared to the other two cultivars suggesting that posttranslational modifications may result in higher H^+ pumping activity in Golden Promise cultivar.

3.4.2 Plant tissue tolerance was a dominating component that has determined the overall plant responses to salinity

The tolerant cultivar Numar maintained root K^+ content at a constant level throughout the eight day salt stress period, while the two other varieties showed a progressive decline in root K^+ content (with the greatest decline in the salt-sensitive NasoNijo) (Fig. 3.3C). This was further corroborated in MIFE experiments measuring NaCl-induced K^+ efflux from barley roots (Fig. 3.4). The rationale behind this experiment is that when Na^+ is absorbed from extracellular space, the membrane gets depolarised and this depolarisation of cell membrane initiates potassium leak as a result of the activation of depolarisation activated outward-rectified potassium channels (KOR) (Chen *et al.* 2007d; Shabala *et al.* 2006). This reduces the K^+ content in the cytosol negatively affecting cell metabolism (Shabala *et al.* 2008) and in turn brings about programmed cell death (Demidchik *et al.* 2010; Shabala 2009). Potassium loss from the epidermal cell in the elongation and mature zone of three barley plants was studied and the highest potassium loss was observed in the salt sensitive cultivar NasoNijo followed by the tolerant cultivars (Numar and GP) in both zones (Fig. 3.4 A, B), reflecting overall tolerance estimated by agronomical (biomass accumulation; Fig. 3.1) and whole-plant physiological (chlorophyll content; Fig. 3.2) characteristics. At the same time, osmotolerance appears to be not central to the overall plant performance under saline conditions. Indeed, the intermediate salt tolerant Golden Promise outperformed the salt-tolerant cultivar Numar, having highest Gs and shoot water content values under saline conditions (Fig. 3.2). This corroborates the point made by Rajendran *et al.*, (2009) that superiority in one salt tolerant component does not guarantee an enhanced overall salt tolerance performance.

Gas exchange (Gs) could be considered as a yield determinant and a valuable tool as a physiological trait that can readily be used as a breeding tool (Lu *et al.* 1998; Richards 2000). Carbon entry and transpiration occurs using open stomata which both helps to increase

photosynthesis and nutrient absorption from the growth media. However, under saline conditions, the stomata tend to close to prevent water loss as it is a scarce resource due to osmotic imbalance. Thus, higher Gs values will ensure better CO_2 assimilation ability (and, hence, higher yield) only when plants have a biochemical and physiological capacity to fully utilise it. This means that higher Gs values must be complemented by higher shoot tissue tolerance, to ensure efficient leaf photochemistry under saline conditions.

To our great surprise, the expression of the barley homologue of *GORK* gene showed high up-regulation in all cultivars. This is counterintuitive, as the opposite effect would be hypothesised due to the very strong correlation between barley salinity stress tolerance and its ability to prevent NaCl-induced K^+ leak from roots (Chen *et al.* 2005; Chen *et al.* 2007b; Chen *et al.* 2007d). These findings, however, are fully consistent with the above notion that transcriptional changes appear to be causally unrelated to functional plant responses. It could be suggested that the post-translational phenomena of assembly might play a role in the regulation of this gene as it is fundamental for the pore formation and electric activity to form tetramer of its α-subunit (Dreyer *et al.* 1997). Furthermore, the availability of the α-subunits and its functional tetrameric assembly is of paramount importance for the cell (Dreyer *et al.* 2004).

Another fact supporting the essential role of tissue tolerance in overall plant performance under saline conditions could be found in kinetics of Na^+ accumulation in the shoot (Fig. 3.3). Consistent with previous reports (Bose *et al.* 2014a; Mian *et al.* 2011; Shabala *et al.* 2010), the tolerant cultivar Numar tends to send more Na^+ to the shoot via the transpiration stream and use it as a cheap osmoticum to maintain shoot turgor, while sensitive variety Naso Nijo delayed this process favouring Na^+ accumulation in the roots (Fig. 3.3A, B). Despite this, Numar plants were capable to maintain higher chlorophyll levels while massive chlorosis was observed in Naso Nijo variety (Fig 3.3). As the volume of the leaf epidermis is small; the

explanation for Na^+ sequestration would be vacuolar compartmentalisation in the mesophyll cells. Consistent with this, salinity treatment caused an increase in the size of palisade parenchyma cells; this increase was much higher in the tolerant barley cultivars (Shabala *et al.* 2010). A similar phenomena is widespread in halophytes (Shabala 2013), species considered to be most tolerant to salt stress. Halophytes also use additional tissue tolerance mechanism such as salt bladders to accumulate excess Na^+ away from the photosynthetic tissue.

3.4.3 Hydroxyl radical-induced K^+ loss is negatively correlated with salinity stress tolerance

In addition to the two above components of the tissue tolerance mechanism, namely better K^+ retention by roots and more efficient vacuolar Na^+ sequestration in shoots, salinity stress tolerance in barley is also correlated strongly with its ability to prevent hydroxyl radical - induced K^+ loss (Fig 3.6). The highest potassium efflux was observed from the salt sensitive cultivar Naso Nijo, followed by Golden Promise (intermediate) and Numar (tolerant). The hydroxyl radical-induced effects were higher in the mature root zone (Fig. 3.6A & B). These findings are consistent with previous reports for root sensitivity to H_2O_2 (Chen *et al.* 2007d; Maksimovic *et al.* 2013) suggesting that sensitivity to oxidative stress is an .essential component of the tissue tolerance mechanism.

An increase in ROS production under salinity stress is attributable to activity of NADPH oxidase, a cell wall-associated peroxidase that generates $O_2^{\cdot}$- by oxidizing NADPH and transferring the electron to oxygen (O_2) (Sagi *et al.* 2006). Accordingly, the regulation of the gene at the transcript level was studied. The relative gene expression at the transcript level of the barley homologue *RBoHF* gene for all of the cultivars was significantly down-regulated but only in leaves but not roots (Fig. 3.8). This down-regulation was least in salt-sensitive NasoNijo, suggesting a possible causal link between NADPH oxidase activity and tissue tolerance mechanisms in salinised mesophyll tissues. However, unlike the leaf, in the root the

transcript abundance was non-significant, except for Golden Promise (Fig. 3.8). The plausible explanation for this could be the post-translational modification such as activation by Ca^{2+} owing to the EF-hand motif for binding Ca^{2+} (Sagi *et al.* 2006).

Potassium efflux induced by hydroxyl radicals is mediated by two major transport systems: (i) non-selective cation channel (NSCC) and (ii) depolarization-activated K^+-selective outward rectifying channel (Demidchik *et al.* 2010; Demidchik *et al.* 2003). High cytosolic K^+ levels are also essential to suppress activity of caspase-like proteases and endonucleases, both in mammalian (Hughes *et al.* 1998, 1999) and plant (Demidchik *et al.* 2010; Shabala *et al.* 2007) systems. Decrease in the cytosolic K^+ pool in plant roots may result in activation of these catabolic enzymes triggering programmed cell death (PCD), especially in sensitive root apex cells (Fig. 3.6).

3.5 Conclusion

In this study, it was possible to show that much of the genes under scrutiny, at the transcriptional level, has lower explanatory value towards the contribution of this gene towards plants adaptive responses to salinity. At the post-translational (e.g. functional) level, tissue tolerance was the dominant mechanism of the salt tolerance. For better tissue tolerance, sodium sequestration, K^+ retention and resistance to oxidative stress all appeared to be crucial. These traits seem to be highly interrelated, as cytosolic K^+ retention is essential for the optimal vacuolar H^+ pump operations (Shabala 2013) required to fuel *NHX* Na^+/H^+ exchanger to enable Na^+ sequestration. Cytosolic K^+ homeostasis, in its turn, is strongly affected by the sensitivity of plasma membrane transporters to ROS and the plant's ability to prevent stress-induced hydroxyl radical accumulation in stressed tissues. Thus, it appears that a major progress in crop breeding for salinity tolerance can be achieved only if these complementary traits are targeted at the same time.

Chapter 4 Expressing AtNHX1 in barley (*Hordeum vulgare* L.) does not improve plant performance under saline condition[3]

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Abstract

Salinity is one of the main causes of yield loss in agricultural crop production worldwide. Numerous attempts have been made to produce salinity tolerant crops by manipulating the expression of genes involved in ion transport; yet, the reported results are often rather controversial. In this work, Arabidopsis *AtNHX1* gene encoding vacuolar Na^+/H^+ exchanger was expressed in barley (*Horduem vulgare* L. cv. Golden Promise) plants grown under saline conditions. Transgenic plants were compared to null segregants for biomass, water content, gas exchange, and Na^+ and K^+ content of the leaf. It was shown that most of the lines expressing *AtNHX1* have no significant difference to null segregants, suggesting the lack of beneficial effect of expressing the *AtNHX1* gene in barley. The lack of phenotype might be

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explained by: (i) low level of activity of vacuolar H^+-inorganic pyrophosphatase and vacuolar H^+-ATPase resulting in an insufficient proton gradient required for vacuolar Na^+/H^+ exchanger; (ii) the inability of transgenic plants to prevent a passive leak of sodium via Na^+ permeable slow activating (SV) and fast activating (FV) channels; (iii) insufficient ATP pool to support H^+ pumping activity; and (iv) the fact that the AtNHX1 protein might not been properly folded, inactive or incorrectly targeted. Overall, this study suggests that, while targeting vacuolar Na^+ sequestration, at least several genes need to be pyramided to ensure the efficiency of the above process. It also suggests the need to verify the proper folding and targeting of the protein in transgenic plants.

Keywords: barley, AtNHX1, Salinity, vacuolar sequestration, vacuolar channels, proton pumping

4.1 Introduction

Saline soils cover 950 million ha, accounting for 10% of the land surface. It has been estimated that 50% (230 million ha) of irrigated land are affected by salt (Ruan *et al.* 2010) and is responsible for an estimated loss of $US27 billion per annum (Qadir *et al.* 2014) . In Australia, 67% of the agricultural land has been estimated to be affected by transient salinity (Rengasamy 2006), costing the Australia farming economy approximately $AUS 1330 million per year (Rengasamy 2002).

Plants have developed numerous mechanisms allowing them to adapt to and tolerate salt stress. Ionic tissue tolerance is one such mechanism. Ion toxicity occurs when normal cellular metabolic processes are disrupted as a consequence of increased ion concentrations. Na^+ ions are the principal cause of ion toxicity caused to cells by salinity stress, resulting in the significant disturbance to cell metabolism, enzymatic activity, and ionic homeostasis (Ahmad *et al.* 2014; Anschütz *et al.* 2014; Shabala *et al.* 2014). To perform well under saline conditions, Na^+ ions should be excluded from shoots, whereas K^+ ions kept at high level ,

thereby maintaining a high cytosolic K^+/Na^+ ratio, especially in leaves (Apse *et al.* 1999; Dubcovsky *et al.* 1996; Gorham *et al.* 1987; Ren *et al.* 2005; Schachtman *et al.* 1989; Serrano *et al.* 2001; Shi *et al.* 2002a). Plants sustain high K^+/Na^+ ratios in the cytosol by expelling Na^+ ions across the plasma membrane and/or by intracellular compartmentalisation of Na^+ ions within the plant vacuole. These processes are crucial for cytosolic Na^+ ion detoxification and for cellular osmotic adjustment, conferring tolerance to salt stress (Blumwald 2000) . Na^+/H^+ antiporters exchange protons for Na^+ ions when moving Na^+ across membranes and are particularly active in the vacuoles of plants, algae and fungi. This type of antiporter is also known to exist in animals, yeasts, bacteria and plants (Blumwald 2000). A gene encoding a Na^+/H^+ exchanger (*NHX1*) has been previously identified as encoding a protein important in reducing the accumulation of Na^+ in the cytosol compartmentalizing Na^+ in prevacuolar and vacuolar compartments (Nass *et al.* 1997; Nass *et al.* 1998). The NHX1 protein is located at the tonoplast and removes Na^+ ions from the cytoplasm. This transporter is energized by the electrochemical potential created by the pumping of H^+ into the vacuole by two proton pumps, vacuolar H^+-inorganic pyrophosphatase (V-PPase, E.C. 3.6.1.1.) and vacuolar H^+-ATPase (V-ATPase, E.C. 3.6.1.3) (Gaxiola *et al.* 2007; Sze *et al.* 1999). The *Arabidopsis thaliana* Na^+/H^+ antiporter gene (*AtNHX1)* was the first plant *NHX* homolog to be cloned (Gaxiola *et al.* 1999). There are six isoforms of *AtNHX* in Arabidopsis with *AtNHX1* and *AtNHX2* having the greatest transcript distribution between tissues and being highly expressed. *AtNHX3* and *AtNHX4* transcript are exclusively found in flowers and roots (Aharon *et al.* 2003; Yokoi *et al.* 2002). *AtNHX5* and *AtNHX6* are believed to be localized in endosomes and functioning in growth and development, salinity tolerance and vesicle trafficking (Bassil *et al.* 2011a; Qiu 2012) Recently, NHX1 and NHX2 were found to transport K^+ into the vacuole giving them a new role in K^+ homeostasis in Arabidopsis (Barragan *et al.* 2012; Bassil *et al.* 2011b)

Previous studies have shown improvement in salt tolerance by expressing *NHX1* in a range of salt excluding plants. The pioneering work by Apse et al (1999) has reported the beneficial effect of over-expressing *NHX1* in Arabidopsis (Apse *et al.* 1999). Improvement in salinity tolerance was then reported for transgenic plants expressing barley *Hv-NHX2* in Arabidopsis (Bayat *et al.* 2011), cotton *GhNHX1* in tobacco plants (Wu *et al.* 2004), Arabidopsis *AtNHX1* in tomato, *Brassica napus* (Zhang *et al.* 2001a; Zhang *et al.* 2001b) and wheat (Xue *et al.* 2004), and *Thellinguella halophile ThNHX1* in Arabidopsis (Wu *et al.* 2009). However, all these studies were conducted on salt-sensitive glycophytes whose "default strategy" is Na^+ exclusion from uptake. Will such beneficial effects be observed in "salt ïncluders", such as barley? In this study we report that merely expressing of Arabidopsis *AtNHX1* in barley has no benefit in increasing plant performance at a whole plant level under saline condition. The possible reasons for the lack of benefit in biomass and other physiological parameters and the implications for breeding salt tolerant plants are discussed.

4.2 Materials and Methods

4.2.1 Plant Material and Growth Condition

The plant material used throughout this study was barley (*Hordeum vulgare* L., cv. Golden Promise). The barley seeds were germinated and grown in 2 L pot filled with University of California (UC) soil mixture (1:1 peat: sand) (Schilling *et al.* 2014). The seeds were watered with reverse osmosis water (RO water) for approximately two weeks until the crop third leaf had fully emerged. Subsequently, NaCl salt was applied for the next five weeks once in two days with 0 mM, 200 mM or 250 mM NaCl. Two *AtNHX1* expressing barley lines and a null segregant were used in the study. For each line there were 6 pots containing 4 plants per pot per treatment. Experiments were conducted between February and April, 2013 in a controlled greenhouse in Adelaide (day/night temperature +25/+10^{o}C, 55-75% relative humidity). Plants were grown under a natural day light.

4.2.2 Cloning of *AtNHX1* and generating transgenic lines

The coding sequence of *AtNHX1* (*At5g27150*) was cloned from cDNA by PCR and ligated into g pCR™ 8/GW/TOPO® TA entry vector. After confirmation of orientation and sequence integrity the transgene was transferred to pMDC32 expression vector using an LR reaction (Gateway Technology, Invitrogene; http//www.invitrogen.com) (Jacobs *et al.* 2007). The expression vector was transformed to *Agrobacterium tumefacience*. Transgenic barley (cv. Golden Promise) expressing *AtNHX1* using the CaMV 35S promoter was successfully generated via Agrobacterium-mediated transformation (Jacobs et al., 2007; Singh et al., 1997). The T_3 transgenic barley lines were used for this study. The control plants used for the study were null segregant lines.

4.2.3 Genotyping of *35S:AtNHX1* in barley

Genomic DNA was extracted from leaf tissue of transgenic and null segregants using Edwards, Johnston and Thomson (1991) method. Forward and reverse primers: 5′-ACT CAT AAG CTA CCT ATT ACC G-3′ and 5′-TTA CTA AGA TCA GGA GGG TTT CTC-3′ were used to amplify a 309 bp amplicon of *AtNHX1* and as a control gene , the *HvVRT2* vernalization gene (GenBank DQ201168) was used and amplified using *HvVRT2*-specific forward primer 5′-CCG AAT GTA CTG CCG TCA TCA CAG-3′ and reverse primer 5′-TGG CAG AGG AAA ATA TGC GCT TGA-3′, which amplified a fragment of 280 bp in size to determine the presence or absence of the gene of interest. Similar PCR conditions were used for both gene of interest and control gene. ImmoMix™ Red (BIOLINE) was used for the PCR (39 cycles of denaturation 94°C for 1 min, annealing 55°C for 1 min and extension 72°C and a final extension of 72°C for 10 min). PCRs contained 2 × reaction of ImmoMix™ Red, 10µM of each forward and reverse primers) 1mg/ml BSA, and 0.5 µL of template DNA and ImmoMix Red is based on IMMOLASE™ DNA Polymerase. Gel electrophoresis was performed using 2% agarose with 5 µL/100 ml Gel Red to visualise the bands.

4.2.4 Determination of transgene expression

Total RNA was extracted using BIOLINE kit. RNA was diluted to equal concentration of 10 ng/µl and subjected to one step RT-PCR using QIAGEN kit. For the amplification of the *AtNHX1* a forward 5′ CCG CAA TTT CGT GAC TAT TAT G 3′ and reverse 5′ GCT TCG TGG TTT AGG TGA G 3′ primer were used with a product size of 309bp. *HvGAP* gene (GenBank EF409629) was used as a positive control (forward primer 5′-GTG AGG CTG GTG CTG ATT ACG-3′ and reverse primer 5′-TGG TGC AGC TAG CAT TTG ACA C-3′; product size of 189 bp). The temperature of the one step RT-PCR was programmed at 50°C for 30 min to activate the reverse transcriptase (RT) and 95°C for 15 min to inactivate the RT and denature the cDNA and 35 cycle of denaturation at 94°C for 1 min, annealing 55°C for 1 min and extension 72°C and a final extension of 72°C for 10 min. The RT-PCR reaction mix contained 5× buffer,10 µM of each forward and reverse primer pairs for the *AtNHX1* and control gene *HvGAP*, water, QIAGEN OneStep RT-PCR Enzyme Mix 2 µL and template RNA (10 ng/µl).

4.2.5 DNA extraction for Southern blot analysis

Plant material was collected in 10 mL tubes, snap frozen in liquid nitrogen and ground to a fine powder by vortexing with three to five sterilised steel balls (Ø 2 mm). The powder was suspended in 1.4 mL of DNA extraction buffer [1 % (v/v) N-Lauroylsarcosine (sigma)-solute to colourless liquid with 50 mg/ml methanol, 100 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 2 % (w/v) polyvinyl-polypyrrolidone (PVPP, Sigma-Aldrich, Castle Hill, Australia), pH 8.5 with HCl] and thoroughly mixed for 1 min before addition of 1.4 mL of phenol/chloroform/iso-amyl alcohol (25:24:1). After a further 15 min mixing, the samples were centrifuged at 3600 g for 10 min. The aqueous phase was transferred into a 2 mL microcentrifuge tube and DNA precipitated with 100 µL of 3 M sodium acetate and 800 µL of isopropanol. The samples were left for 1 hr at RT before centrifugation at 10,000 g for 10 min.

The supernatant was removed and the pellet washed in 1 mL 70 % (v/v) ethanol. The pellet was air dried and resuspended in 100 µL of ddH2O containing 10 mg/mL of RNase (Thermo Fisher Scientific Australia Pty Ltd., Scoresby, Australia). The obtained DNA is of high purity and can be used for subsequent Southern Blot analysis.

4.2.6 Southern blot analysis

The DNA samples for the *AtNHX1* lines (*35S:AtNHX1-1* and *35S:AtNHX1-2)*, Null segregant and Golden promise (WT) were subjected to Southern blot analysis. The genomic DNA (1 µg) were digested with 20 units of *BamHI* (which cuts once inside the gene *AtNHX1* and in between the gene and the 35S promoter) and *HindIII* (which cuts only once in the vector, at the start of the promoter). An alkaline Southern blot was assembled using a Hybond-N^+ membrane (Amersham) according to manufacturer's instructions. DNA was fixed to the membrane by cross-linking in a UV Stratalinker 2400 (Stratagene). Probe DNA was produced by PCR using primers to amplify fragment of the *hpt* coding sequence. The probe was labelled with 32P α-dCTP prior to hybridisation. The blot was imaged using a Typhoon 9200 Phosphorimager (Amersham).

4.2.7 Biomass Measurements

The fresh weight of the above ground biomass was recorded at the end of the study period. The dry weight of each sample plants was measured, after drying the plants at 65^0C for 72 hrs in an oven. As an indicator of biomass, plant height was recorded at the end of the study period.

4.2.8 Na^+ and K^+ content of the leaf

At the end of the salt application, the third leaf of barley plants was removed and oven dried as described above. A leaf aliquot of 0.3 g was then digested in 1% nitric acid by heating at 85 ^{0}C for 4 hrs. on a hot block (Environmental Express, Mt Pleasant, SC, USA). The solution in the digested leaf was diluted with milliQ water in a dilution factor of 1:20. The Na^+ and K^+

content were measured by a flame photometer model 420 (Sherwood Scientific LTD, Cambridge, UK).

4.2.9 Gas exchange and water relations

The stomatal conductance (G_s) was measured from the third leaf after the culmination of the NaCl treatment on a sunny day during the day times when the plants are fully transpiring. For measuring the G_s, leaf porometer Model SC-1 (Deacagon device INC, WA, Australia) was used. The measurements were taken consistently from one-third from the leaf tip of the third leaf in all samples. The relative water content (RWC) was calculated from the fresh and dry weight of the plants by using the formula:

$$RWC = \frac{FW-DW \times 100}{FW}$$

4.2.10 Statistical analysis

The data was analysed using student's t-test method and the significant level of mean difference was compared at probability value of $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***) using a statistical software SPSS version 22 (IBM support portal, USA).

4.3 Results

4.3.1 Growth response of barley plants expressing *AtNHX1*

The presence of the *AtNHX1* transgene in the transgenic barley was confirmed by PCR. PCR analysis showed that the *AtNHX1* gene was present in *35S:AtNHX1-1* and *35S:AtNHX1-2* lines and absent in both the null and Golden Promise (GP) (Fig. 4.1a). RT-PCR was performed to confirm that the transgene was expressed in the transgenic lines and not in either of the nulls or the wild type (Fig. 4.1b). Southern blot analysis was done to check the number of inserts of *AtNHX1* in the transgenic lines. *35S:AtNHX1-1* had a single insert while *35S:AtNHX1-2* had three inserts. Both the null line and Golden Promise were found to have no insert (Fig. 4.2). The two barley *AtNHX1* expressing lines, a null segregant and Golden Promise were treated with 200 mM and 250 mM NaCl for five weeks after seedling establishment. *AtNHX1* expressing lines did not show any obvious phenotype and did not gain any advantage in growth and development compared to the wild type or null barley plants under both control and saline conditions (Fig. 4.3). The biomass of all plants was significantly reduced after the addition of salt. Moreover, the fresh weight of *AtNHX1-2* line was 15% lower compared to the null segregant (significant at $P \leq 0.01$). The other transgenic line didn't show significant (at $P \leq 0.05$) reduction in fresh weight compared to the null segregants (Fig. 4.4a). No significant change in dry weight was observed in transgenic lines (Fig. 4.4b). Taken together, these results suggest that expressing *AtNHX1* in barley does not improve plant phenotype under saline conditions.

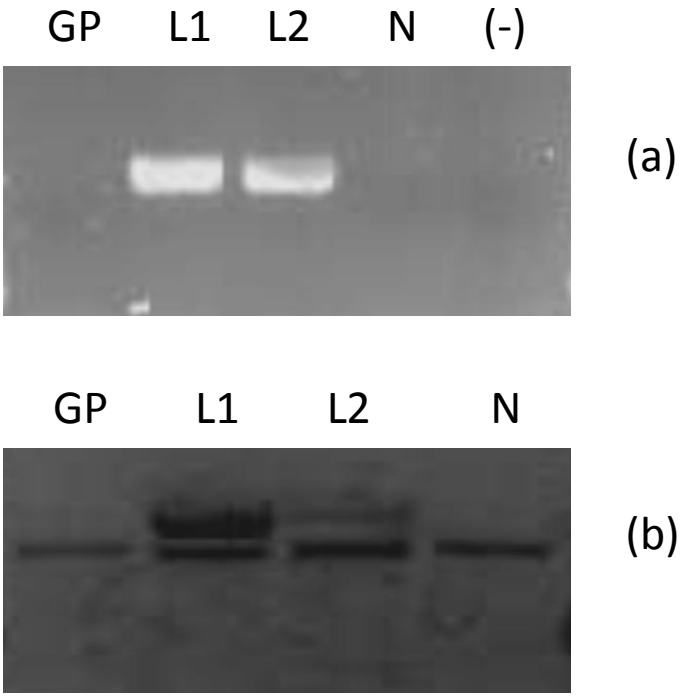

Figure 4.1 Molecular analysis of *35S:AtNHX1* lines in barley (a) Confirmation of transgene presence by PCR. GP (Golden Promise), L1 (*35S:AtNHX1-1*), L2 (*35S:AtNHX1-2*), N (null line), (-) negative control. (b) Confirmation of transgene expression in transgenic lines by RT-PCR. A multiplex reaction using primers for *AtNHX1* (upper band) and the control gene *Hv-GAP* (lower band) was performed.

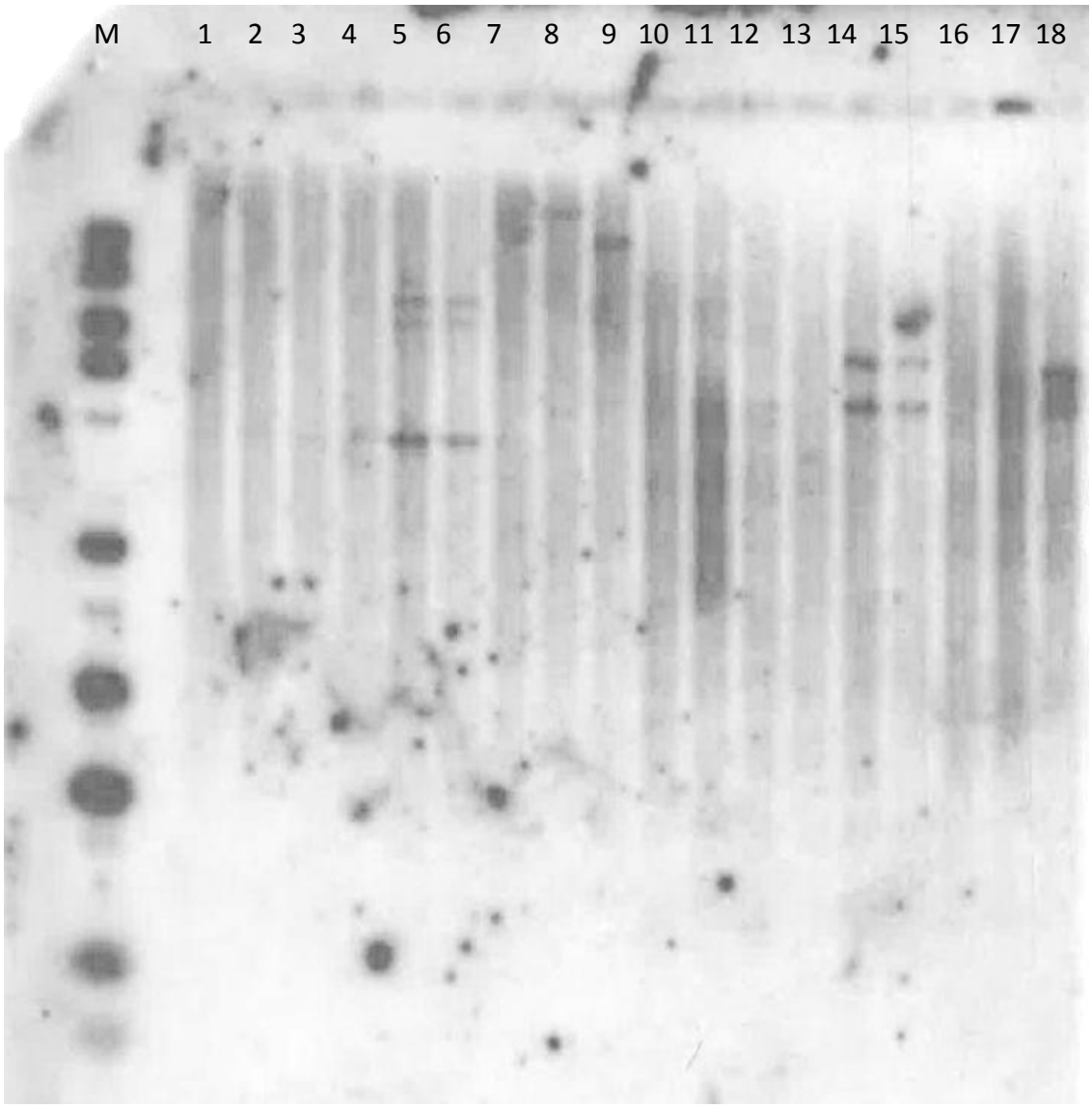

Figure 4.2 Southern blot analysis of two transgenic lines, a null line and Golden Promise. The Genomic DNA was digested with BamHI and HindIII restriction enzymes and probed by hygromicin DNA. The lanes 1-2 and 10-11 correspond to Golden Promise. Lanes 3-4 and 12-13 correspond to null line. Lanes 5-6 and 14-15 correspond to *35S:AtNHX1-2*. Lanes 7-8 and 16-17 correspond to *35S:AtNHX1-1* with BamHI and HindIII digestion, respectively. Lane 9 and 18 are positive controls.

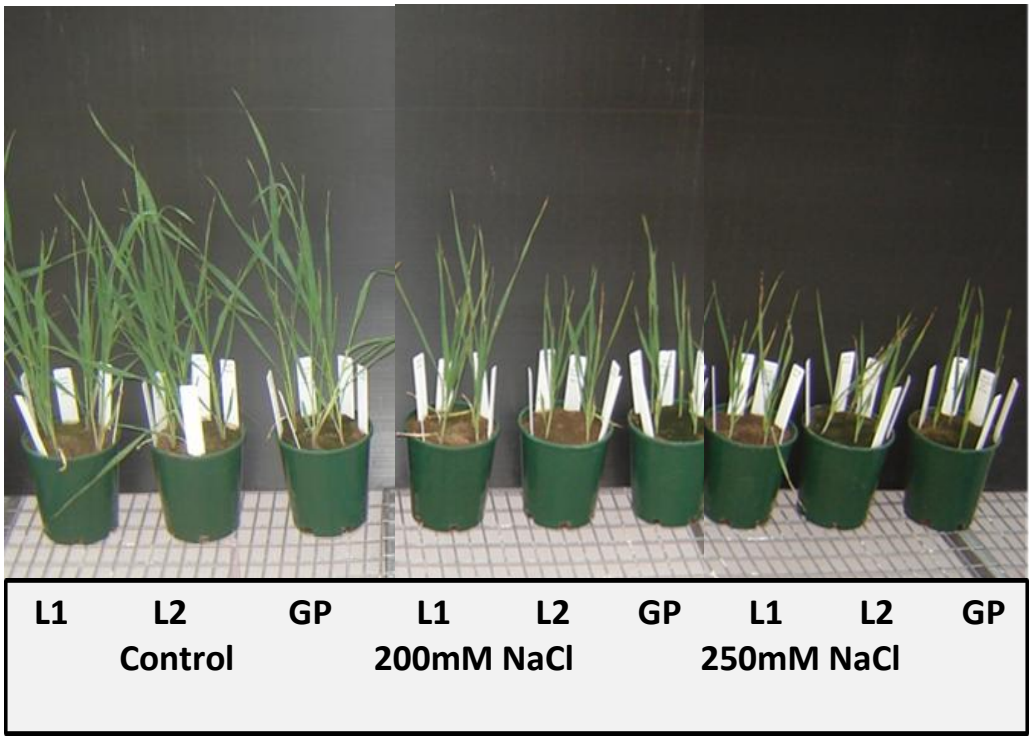

Figure 4.3 Growth and development of three barley *35S:AtNHX1* lines and non-transgenic control cultivar Golden promise (GP) treated with 0 mM, 200 mM NaCl and 250 mM NaCl for five weeks after seedling establishment. *35S:AtNHX1-1* corresponds to line L1, *35S:AtNHX1-2* to L2, and GP to Golden Promise.

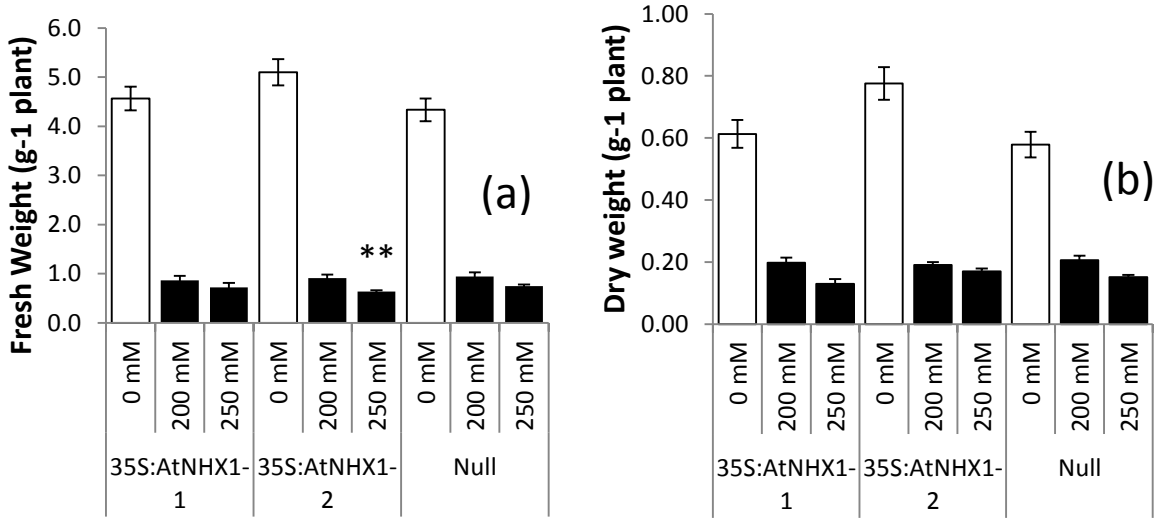

Figure 4.4 Fresh weight (a) and dry weight (b) of two barley *35S:AtNHX1* lines and the null line. Results are mean ± SE (n=21-24). Statistical significance designated as $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

4.3.2 Expressing *AtNHX1* in barley gives no advantage to either enhanced gas exchange or relative water content

The stomatal conductance of all lines tested was reduced by approximately 60 to 70% after treatment with NaCl. Expressing *AtNHX1* in barley did not result in improvements in stomatal conductance under salt stress (Fig. 4.5a). Relative water content of all salt grown lines was significantly reduced when compared to those grown in the absence of salt. The *35S:AtNHX1-2* line showed a 4% reduction in RWC (significant at $P \leq 0.001$) compared to the null segregant. The *35S:AtNHX1-1* line has non-significant difference with the null segregant. Taken together, these results suggest the lack of beneficial, or even some detrimental (as in line *35S:AtNHX1-2*) effects of *AtNHX1* expression for gas and water relations in barley (Fig. 4.5a & b).

4.3.3. Expressing *AtNHX1* in barley has not resulted in increased chlorophyll content

There was no significant difference in chlorophyll content in *35S:AtNHX1-1* when compared to the null line. In *35S:AtNHX1-2* line, a small but significant (at $P \leq 0.05$) decline in chlorophyll content was observed between as compared with the null (Fig. 4.6). This suggests that chlorophyll content could not be positively influenced by expressing *AtNHX1* in barley.

4.3.4 Plants expressing *AtNHX1* showed increased shoot Na^+ and K^+ accumulation

Leaf Na^+ accumulation increase substantially 3.5-4.5 fold higher in all lines after the addition of NaCl. The *35S:AtNHX1-2* line showed 22% increase in leaf Na^+ accumulation compared to the null segregant (significant at $P \leq 0.001$). The other transgenic line *35S:AtNHX1-1* also showed slight (14%) but not significant increase in leaf Na^+ content (Fig. 4.7a). Similarly, K^+ accumulation in the shoot was approximately 30% higher (significant at $P \leq 0.001$) for the *35S:AtNHX1-2* line compared to the null segregant, and marginally higher (8%; not significant at $P \leq 0.05$) shoot K^+ accumulation was measured in *35S:AtNHX1-1* (Fig. 4.7b).

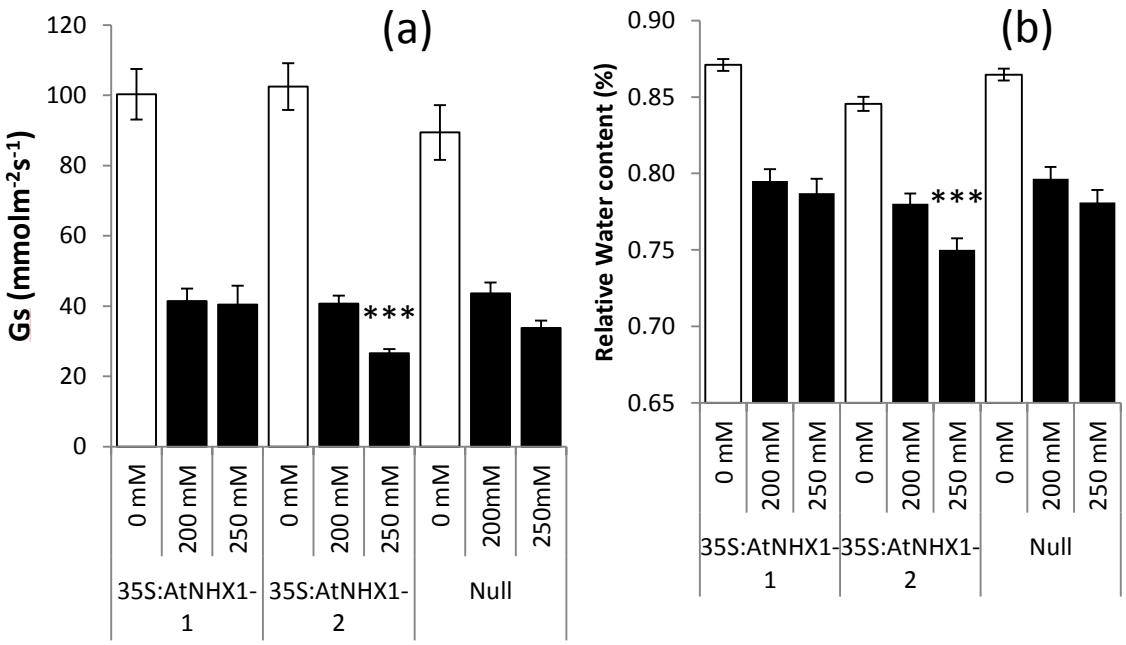

Figure 4.5 (a) Stomatal conductance of two barley *35S:AtNHX1* lines and null lines. Results are the mean ± SE (n=21-24) (b) Relative water content of two barley *35S:AtNHX1* lines and a null. Results are mean ± SE (n=21-24). Statistical significance designated as $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

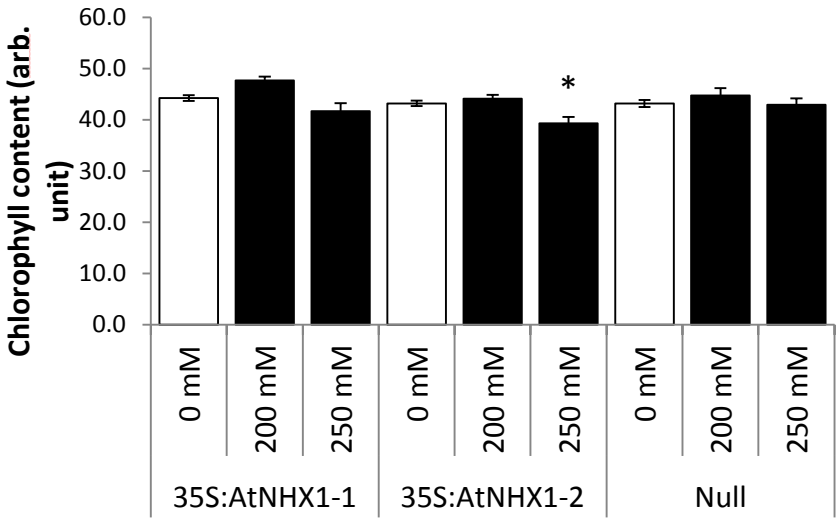

Figure 4.6 Chlorophyll content (arb. unit) of two *35S:AtNHX1* barley lines and a null line were exposed to 0 mM, 200mM and 250mM NaCl for five weeks after seedling establishment. Results are the mean ± SE (n=21-24). Statistical significance designated as $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

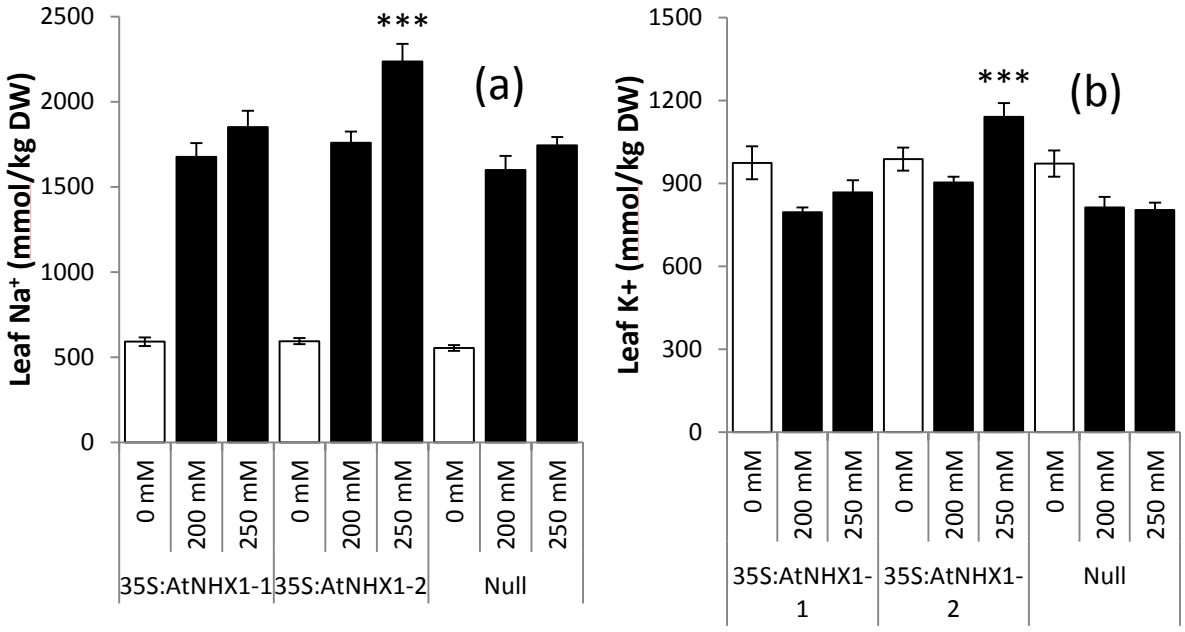

Figure 4.7 Leaf Na^+ (a) and K^+ (b) content of two *35S:AtNHX1* barley lines and null (used as control) exposed to 200mM and 250mM NaCl for five weeks after seedling establishment. Mean ± SE (n=21-24). Mean ± SE (n=21-24). Statistical significance designated as $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***)

4.4 Discussion

4.4.1 Expressing AtNHX1 alone in barley has no advantage for biomass

The biomass of the *AtNHX1* expressing barley lines has shown non-significant change compare to the null segregants, suggesting that the expression of *AtNHX1* in has no beneficial effect in increasing biomass. Moreover, *AtNHX1-2* line even showed a small but significant reduction in fresh weight under 250 mM salt treatment (Fig. 4.4a & b). Several possible reasons may explain this lack of beneficial effects. One of them is a possible misfolding of the AtNHX1 protein which would result in an inactive protein and no improvement in phenotype. Another reason might be the mistargeting of the protein to other membranes, where it is not usually associated with. Constitutive over-expression of genes encoding membrane proteins is known to often result in a large amount of protein produced, leading that protein appearing on other membranes. For example over-expression of *AVP1* can result in the vacuolar H^+-PPase

being targeted to the plasma membrane (Gaxiola *et al.* 2012; Li *et al.* 2005). Thirdly, the lack of phenotype may be due to NHX1 protein not undergoing the required post-translational modifications, such as by a kinase, to activate its transporter capabilities (Neuhaus *et al.* 2014; Weinl *et al.* 2009). It has been reported that NHX proteins may be targeted by the CBL-CIPK calcium signalling pathway, similar to the regulation of AtSOS1 by the kinase AtSOS2 (AtCIPK24) (Qiu *et al.* 2004).

Apart from issues with the production of an Arabidopsis protein in barley there are other physiological reasons as to why no phenotypic difference was observed between the transgenic lines and nulls. First, the 'pump and leak concept' explained for plasma membrane of algal cell (Brummer *et al.* 1985) also works for plasmalemma and tonoplast of higher plant (Hedrich *et al.* 2011). Vacuolar Na^+ may back-leak to the cytosol via Na^{+-} permeable SV (slow activating) and FV (Fast activating) channels, and it was shown that reduced activity of these channels confer salt tolerance in halophyte species (Bonales-Alatorre *et al.* 2013b; Hedrich *et al.* 1987). SV channels conduct both mono- and divalent cations indiscriminately based on Ca^{2+} and voltage dependence and FV channels conduct monovalent cations including K^+ (Pottosin *et al.* 2014). It was suggested earlier that, to achieve efficient vacuolar Na^+ sequestration, increased NHX activity should be accompanied by efficient control of SV and FV channels permeability (Bonales-Alatorre *et al.* 2013b; Shabala 2013). This may be not the case in this study. Second, the proton pumps V-ATPase and V-PPiase are responsible for developing proton motive force to move ions into the vacuole (Gaxiola *et al.* 2007; Sze *et al.* 1999), and their high activity is essential for efficient vascular sequestration of Na^+ by NHX (Shabala 2013). Thus, it may appear that in barley the intrinsic tonoplast H^+-pumping activity is not sufficient to drive any "extra" NHX, and concurrent expression of NHX transporters and H^+ pumps is required to confer salinity tolerance. Third, vacuolar H^+-ATPse pumping require ATP and, even plants may have enough functional AHA proteins encoding this

pumping, they may be simply short of ATP to make them functional. Forth, the reduced K^+ pool in the cytosol due to its sequestration in the vacuole would seek compensation by production of osmolytes to prevent dehydration. However, the mitigating effect of osmolytes for loss of K^+ from the cytosol in tolerant barley genotypes is less pronounced (Chen *et al.* 2007a) and the genetic background used for this study is a comparatively tolerant barley cultivar (Adem *et al.* 2014).

Taken together, it appears that transgenic barley expressing *AtNHX1* requires pyramiding with genes that will energize the tonoplast membrane. It was shown earlier that the co-expression of *Suaeda salsa* Na^+/H^+ antiporter (*SsNHX1*) and *AVP1* has shown higher salinity tolerance in rice than the expression of *SsNHX1* alone (Zhao *et al.* 2006) and required a kinase gene to assist in the activity of the NHX protein. Also, its deemed necessary considering to control back leak of Na^+ in the positive membrane potential sensing Na^+ permeable SV and FV channels (Shabala 2013).

4.4.2 Barley plants expressing *AtNHX1* alone has shown no improvement in water relation and gas exchange characteristics

Stomatal conductance is one of physiological trait that is considered as a yield determinant (Lu *et al.* 1998; Richards 2000). Control of stomatal conductance to balance for optimum transpiration rate and water retention is of paramount importance for a plant growth under salt stress (Munns 2002). Cognizant to these facts, there was no increase in stomatal conductance in *35S:AtNHX1-1* line and even there was a significant reduction $P \leq 0.001$ in *35S:AtNHX1-2* line compared to the null segregant. Similar reduction was observed in the fresh weight and RWC. In previous experiment Golden Promise showed a better water retention under salt stress (Adem *et al.* 2014), however, in this experiment it was not possible to improve water retention further for Golden Promise. Presumably a leaky system could be assumed for the Na^+ and K^+ ions which are used as osmoticum for maintain cell turgour, stomatal aperture and

gas exchange and ultimately water relation of the plant where the factors are crucial for adaptation under water stress (Anschütz *et al.* 2014; Mengel *et al.* 1982). Therefore, for obtaining improvement in water relation and gas exchange using AtNHX1 expression in barley has to take into consideration all factors involved in efficient sequestration of ion and proper feedback mechanisms in ion homeostasis which are discussed in this paper.

4.4.3 Sodium and Potassium accumulated in the leaf of barley plants expressing AtNHX1

The Na^+ content in the leaf of the barley plants expressing *AtNHX1* has increased significantly ($P \leq 0.001$) as shown in *35S:AtNHX1-2* line in comparison to null segregants. The two possible scenarios may be higher pumping of Na^+ into the vacuole and hence, higher Na^+ in the shoot or the pleotropic alterations of native gene expression in barley due to the expression of the Arabidopsis transgene which may have unforeseen effects on abundance and activity of proteins involved in Na^+ transport in a plant. Alterations in native gene expression related to ion transport were observed in transgenic rice, after cell type specific expression of *AtHKT1;1* in root cortical cells, which were hypothesized to alter Na^+ transport through the transgenic plants (Plett *et al.* 2010a). Mis-expression of *AtNHX1* in barley may have altered genes encoding proteins which result in a higher translocation of Na^+ to the shoot. Similarly, leaf K^+ content of the *35S:AtNHX1-2* was shown to be higher compared to the null segregants. This may be attributed to the K^+/H^+ transport activity of the *NHX* protein reported recently (Barragan *et al.* 2012; Bassil *et al.* 2011b; Leidi *et al.* 2010; Rodriguez-Rosales *et al.* 2008).The lower K^+ level in the cytosol due to its transport to the vacuole may be one reason for lack of phenotype in the barley plants expressing *AtNHX1*. The *AtNHX1* is not only a Na^+ transporter but also K^+ as cited above. The AtNHX1 transports K^+ to the vacuole reducing the cytosol K^+ pool which is detrimental as a result counteracting the beneficial effect of the protein, therefore, no benefit in performance under salt stress. In addition, the salt induced

repression of *HAK* as shown in *LeHAK5* repression of expression under salt growth condition (Nieves-Cordones *et al.* 2008) which may be considered as other factor for salt tolerance by limiting K^+ uptake under salinity stress.

Taken together, the lack of phenotype might be hypothesized due to a number of reasons: (i) low level of activity of vacuolar H^+-inorganic pyrophosphatase (V-PPase, E.C. 3.6.1.1.) and vacuolar H^+-ATPase (V-ATPase, E.C. 3.6.1.3) resulting in an insufficient proton gradient required for vacuolar Na^+/H^+ exchanger; (ii) the inability of transgenic plants to prevent a passive leak of sodium via Na^+ permeable slow activating (SV) and fast activating (FV) channels; (iii) insufficient ATP pool to support H^+ pumping activity; and (iv) the fact that the NHX1 protein might not been properly folded, inactive or incorrectly targeted. Furthermore, the above mentioned notions can be strengthened by observing the contribution of ectopically expressed *AtNHX1* to endogenous Na^+/H^+ and K^+/H^+ antiport activities in the vacuole of control plants, which could be intrinsically high considering that barley is a salt inculder and cation/proton exchange activity in the transgenic in comparison to control plants.

Chapter 5 Expressing Arabidopsis thaliana V-ATPase subunit C in barley (Hordeum vulgare L.) improves plant performance under saline condition by enabling better osmotic adjustment [4]

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Summary

Salinity is a global problem affecting agriculture resulting in an estimated $27Bln per annum loss revenue. Overexpressing of vacuolar ATPase subunits has been shown to be beneficial in improving plant performance under saline conditions. Most studies, however, have not shown if there is an improvement on grain yield, and/or investigated the physiological mechanisms behind the improvement in plant growth. In this study, we constitutively expressed *Arabidopsis Vacuolar ATPase subunit C* (*AtVHA-C*) in barley. Transgenic plants were assessed for agronomical and physiological characteristics, such as dry biomass, leaf pigment content, stomatal conductance, grain yield, and leaf Na^+ and K^+ content, when grown with either 0 or 300 mM NaCl. When compared to non-transformed barley, the *AtVHA-C* expressing barley lines have a smaller reduction in both biomass and grain yield. The ability of the transgenic lines to maintain higher stomata conductance, resulting from an increase in

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Na^+ and K^+ accumulation in the leaf which could be used for osmotic adjustment rather than the synthesis of energy expensive organic osmolytes.

Key words: vacuolar sequestration; osmotic adjustment; salinity stress tolerance; sodium; potassium; organic osmolytes

5.1 Introduction

With the world population estimated to exceed 9.3 billion by 2050, there is a shift towards agricultural production systems using of marginal saline lands (Panta *et al.* 2014; Shabala 2013). The problem is further aggravated by the reduction in precipitation in subtropical areas, which results in farmers irrigation with brackish and low quality water, as the good quality water is reserved for drinking (Barrett-Lennard *et al.* 2010; Munns *et al.* 2008). The estimated cost of salinity-induced loss to crop production is believed to be in excess of $27 Bln per year (Qadir *et al.* 2014). Improving performance of crop plants so that they produce a better yield under high saline conditions is of paramount importance for using these marginal lands (Munns 2002; Roy *et al.* 2013).

Soil salinity reduces the amount of available water requires plants to make osmotic adjustments to grow in these conditions. Inorganic ions such as K^+, Na^+ and Cl^- play an essential role in this process, contributing to 80-95% of the cell sap osmotic potential in halophytes (Glenn *et al.* 1999) and to 50-70% in glycophytes, such as barley and wheat (Chen *et al.* 2007b; Cuin *et al.* 2010). However, excess Na^+ in the cytosol is toxic to cellular processes, regardless of whether plants are halophytes or glycophytes (Flowers *et al.* 2008). Therefore for Na^+ to be an effective osmolyte it must be sequestered in the vacuole. Three components are essential for the vacuolar storage of Na^+: establishment of the electrochemical gradient necessary to pump Na^+ into the vacuole; Na^+ transport across the tonoplast into the vacuole; and Na^+ retention within the vacuole (Shabala 2013). Tonoplast Na^+/H^+ antiporters such as members of the Na^+/H^+ exchanger (NHX) family have been shown to be important in

the loading of Na^+ into the vacuole (Apse *et al.* 2007; Barkla *et al.* 1995; Blumwald *et al.* 1985; Gaxiola *et al.* 1999). There are numerous NHX proteins which are expressed in different tissue- and stress-dependent manner (Rodriguez-Rosales *et al.* 2008). Proteins such as NHX rely on a proton gradient between the vacuole and the cytoplasm which is generated by two types of tonoplast localized proton pumps, namely vacuolar ATPase (*VHA*) (Krebs *et al.* 2010; Vera-Estrella *et al.* 1999; Wang *et al.* 2001) and vacuolar pyrophosphatase (V-PPase) (Guo *et al.* 2006; Parks *et al.* 2002; Vera-Estrella *et al.* 2005). Lastly, to retain sequestered Na^+ in the vacuole, preventing it from leaking back into the cytosol efficient control of expression and activity of sodium permeable slow (SV) and fast (FV) vacuolar channels is required (Bonales-Alatorre *et al.* 2013a; Bonales-Alatorre *et al.* 2013b; Pantoja *et al.* 1989; Shabala *et al.* 2011a).

Many studies investigating whether improvements in vauolar Na^+ sequestration can improve salinity tolerance, usually overexpression a single *NHX* gene. Results ranged from highly positive (Apse *et al.* 1999; Bayat *et al.* 2011; Zhang *et al.* 2001a; Zhang *et al.* 2001b) to no yield improvement or even detrimental effects (Adem *et al.* 2015). A better approach may be in manipulating the expression of genes encoding proteins which set up up the necessary vacuolar conditions for effective Na^+ sequestraion, such as manipulation of the expression of *V-ATPases* and *V-PPases*.

Overexpression of the Arabidopsis vacuolar H^+-PPase (*AVP1*) has conferred both salinity and drought tolerance in Arabidopsis (Gaxiola *et al.* 2002; Gaxiola *et al.* 2001) and overexpression of H^+-PPase from *Suaeda sala* improved salinity tolerance in Arabidopsis (Guo *et al.* 2006). Consistent with these observations, Zhao *et al.*, (2006) showed that the co-expression of the *Suaeda salsa* Na^+/H^+ antiporter (*SsNHX1*) and *AVP1* conferred higher salinity tolerance in rice than the expression of *SsNHX1* alone.

H^+-PPases are often considered a supporting mechanism to V-ATPases in the acidification of the vacuole (Maeshima 2000). Hence, investigating whether greater improvements in vacuolar storage of Na^+ and in salinity tolerance can be made by manipulatng the abundance of V-ATPases is important.

Unlike V-PPases, vacuolar H^+-V-ATPase (*VHA*) are made of several subunits, encoded by different genes. Thismakes overexpressing the entire protein difficult. Studies have investigated, however, whether manipulation of the expression of genes encoding specific V-ATPase subunits can improve plant growth under saline conditions. Expressing the V-ATPase c1 subunit from the halophyte grass *Spartina alterniflora* improved rice growth uder saline conditions (Baisakh *et al.* 2012). However, the beneficial effect was reported only for vegetative plant growth but not grain yield. Interestingly, the observed effect was explained by the early closure of the leaf stoma and reduced stomata density (Baisakh *et al.* 2012) during salt stress, which is not consistent with the transgenic plants increases their biomass when they have reduced capacity to assimilate CO_2. More recently, genes encoding individual V-ATPase subunits in wheat line were expressed in Arabidopsis (He *et al.* 2014). While improvements in the salt tolerant of seedling transgenic Arabidopsis plants were observed, no insightes into the mechanisms behind the imporved tolerance were reported (He *et al.* 2014). Similar results were reported when wheat *V-ATPase subunit B* (Wang *et al.* 2011) and *subunit E* (Zhao *et al.* 2009) were expressed in Arabidopsis and when the *V-ATPase subunit c* (*VHA-c*) from *Limonium bicolor* was expressed in tobacco (Xu *et al.* 2011).

All the above examples come from salt-sensitive species (rice; Arabidopsis; tobacco) that predominately rely on sodium exclusion to achieve their tolerance (Munns *et al.* 2008). There are other species, such as barley, which will still predominately exclude the majority of the Na^+ in the soil from the shoot are better able at accumualting higher concentrations of Na^+ in the shoot, relying on Na^+ to achieve their osmotic adjustment under saline conditions

(Shabala *et al.* 2010). The questions remains as to what extent overexpression of *V-ATPase* subunits may enhance salinity stress tolerance in barley, and the mechanisms behind any beneficial effect observed.

In this study, we generated lines of barley expressing the Arabidopsis *V-ATPase subunit C* (*VHA-C*) and assessed their physiological and agronomical performance under saline conditions. V-ATPase subunit C was choosen as this is on peripheral of the V1 complex, which has a function in ATP hydrolysis (Perez-Sayans *et al.* 2012; Sze *et al.* 2002), and can directly enhance the activity of the V-ATPase. Barley lines expressing the *AtVHA-C* gene were found to have smaller salinity driven reductions both biomass and grain yield compared to non-transformed wild type plants. These beneficial effects may be linked to the improved ability of the transgenic lines to maintain higher stomata conductance, resulting from increased Na^+ and K^+ accumulation in the leaf which contributed to osmotic adjustment and made plant less reliant on *de novo* synthesis of organic osmolytes.

5.2 Experimental procedures

5.2.1 Generating transgenic barley plants expressing *VHA-C* (Arabidopsis V-ATPase Subunit C)

The coding sequence of *V-ATPase subunit C* (*At1g12840*) was amplified from the cDNA of *Arabidopsis thaliana* (ecotype Col-0) using primer pairs, 5′ -AGA GAC TCG TAA ACA AGA G -3′ and 5′ -CAG CCA TGG CTC CTG CA -3′ and a high fidelity DNA polymerase. The amplified sequence waas introduced into the *pCR8/GW/TOPO TA* Gateway enabled entry vector (Invitrogen). The *V-ATPase C* was then recombined into a *pMDC32* destination vector Gateway LR® recombination reaction (Invitrogen, Carlsbad, CA, USA) (Curtis *et al.* 2003), 3′ of a *CaMV 35S* promoter. The *VHA-C* pMDC32 vector was transformed into the barley, *Hordium vulgare*, cultivar WI4330 (kindly supplied by the University of Adelaide Barley Breeders. the by the *Agrobacterium tumefaciens*- mediated

transformation method using the ACPFG (University of Adelaide) transformation facility. After antibiotic selection, the transformed plantlets were regenerated on soil (Jacobs *et al.* 2007; Singh *et al.* 1997) to produce T_1 seed. After one round of seed multiplication the T_2 plants were grown for experiment in the University of Tasmania.

5.2.2 Plant growth conditions

Four *VHA-C* expressing (*35S:VHA-C*) T_2 lines. described here as OE1, OE2, OE3 and OE4, and one non-transfored WI4330were grown on 2L pot containing potting mix. The potting mix was composed of 70% composted pine bark; 20% coarse sand; 10% sphagnum peat; Limil at 1.8 kg/m3, dolomite at 1.8 kg/m3). The plant nutrient balance was maintained by adding the slow release Osmocote Plus™ fertilizer (at 6 kg/m3), plus ferrous sulphate (at 500 g/m3) (Bonales-Alatorre *et al.* 2013a). The plants were grown in a glasshouse with regulated temperature (24^oC-20^oC day/15^oC night) for approximately 2 weeks (until the 3rd leaf stage) until the seedling established. Then salinity treatment was administered by adding 300mM NaCl with irrigation water until maturity. The experiment was conducted between January and April 2014. Plants were grown in 4L pots, with three plants per pot and 4 to 5 pots per treatment/genotype.

5.2.3 DNA extraction and PCR analysis

Barley genomic DNA from the above lines was extracted using (Edwards *et al.* 1991) method. First, the presence of DNA and the PCR condition was confirmed using a control gene, *Hv-VRT2* vernalization gene (GenBank DQ201168) which was amplified using *Hv-VRT2*-specific forward primer 5′-CCG AAT GTA CTG CCG TCA TCA CAG-3′ and reverse primer 5′-TGG CAG AGG AAA ATA TGC GCT TGA-3′, which amplified a fragment of 280 bp in size. The *VHA-C* gene was detected using a- forward primer 5′-AGA GAC TCG TAA ACA AGA G -3′ and reverse primer 5′-CAG CCA TGG CTC CTG CA -3′, which amplified a fragment of 299 bp in size. PCRs contained 2 × reaction of ImmoMix™ (BIOLINE), forward

and reverse primers (10 µM each), BSA (1 mg/ml), MilliQ water and 0.5 µL of template DNA. The PCR conditions used were similar for both the *VRT2* and the *VHA-C* (95°C for 10 min, followed by 1 min at 94°C, 1 min at 55°C and 1min at 72°C, repeated for 35 cycles, with a final extension of 72°C for 10min). PCR products were visualised by gel electrophoresis using a 2% agarose with 5µL/100ml Gel Red Nucleic acid stain (BIOTIUM) and the image was taken using gel doc (Molecular imager, Gel Doc^{TM} imaging system, BIORAD).

5.2.4 RNA extraction and gene expression analysis

Total RNA was extracted from leaf tissue using BIOLINE plant Isolate II RNA kit (www.bioline.com/isolate). The RNA was diluted to 10 ng/µl and subjected to a RT-PCR with a master mix containing components of 5 × buffer, dNTP (10 mM), water and enzyme mix (*Taq* polymerase and reverse transcriptase), forward and reverse primers for *VHA-C* and *HvGAP* (*VHA-C* gene: forward primer 5′-AGA GAC TCG TAA ACA AGA G -3′ and reverse primer 5′-CAG CCA TGG CTC CTG CA -3′,; and *Hv-GAP*: forward primer 5′-GTG AGG CTG GTG CTG ATT ACG-3′ and reverse primer 5′-TGG TGC AGC TAG CAT TTG ACA C-3′) and a template RNA of 10 ng/ul. Reverse transcription was performed at 50°C for 30 min, followed by PCR (initial PCR activation 95°C for 15 min, then 35 cycles of 94°C for 1min, of 55°C for 1 min and 72°C for 1min, before a final extension 72°C for 10min). The PCR product was visualized by gel electrophoresis using a 2% agarose gel containing 5 µL/100ml of Gel Red Nucleic acid stain (BIOTIUM) using gel doc (Molecular imager, Gel Doc^{TM} imaging system, BIORAD).

5.2.5 Biomass and grain yield

As grain yield was required, only the dry weight of the plants (not fresh weights) was recorded at the end of experiment, alongside with plant height. The number of seeds per plant and their total weight were recorded. The sample size for biomass and yield data for the salt treated plants range from 8 to 15. The number of chlorotic and necrotic leaves per plant was

also recorded after 5 weeks of salt treatment. For chlorotic leaves, the partially yellow leaves per plant was counted and presented as chlorotic leaves. For necrotic leaves, the dead leaves per plant was counted and presented as necrotic leaves.

5.2.6 Pigment content and stomatal conductance

Relative leaf chlorophyll content was measured using Minolta Chlorophyll Meter SPAD-502 (Konica Minolta, Osaka, Japan). Measurements were taken from the third true leaf, at a position about one quarter of the length of the leaf from the leaf tip. The same leaf/position was used to measure stomatal conductance (Gs) from control and salt treated plants. These measurements were conducted using Decagon leaf porometer (Decagon Devices Inc., WA, Australia), under constant light conditions (artificial light of 150 μmol m^{-2} s^{-1}) between 11 am and 3 pm.

5.2.7 Na^+ and K^+ content

The third leaf from both salt treated and control plants were excised after five weeks of salt treatment for Na^+ and K^+ ion content measurement. Leaves were dried in the oven at 65°C for 72 hrs. A subsample of a known weight (about 0.2 g) was then digested in 5ml of 65-70 % of HNO_3 and 2ml of 30% of H_2O_2 using a microwave digester (MDS-2000, CEM corporation, Matthews, NC,USA). The digested samples were diluted 1:5 using distilled water. The Na^+ and K^+ ion content was measured using a flame photometer (MODEL PFP7-Flame photometer, JENWAY, Bibby Scientific Ltd, UK). The concentration of the ion content was calculated as mmol kg^{-1} DW taking aliquot weight and dilution factors into consideration.

5.2.8 Sap osmolality measurements and calculation of contribution of inorganic ions towards leaf osmotic adjustment

After five weeks of treatment with 350 mM NaCl, third leaf of the barley transgenic and WT plants harvested and stored at -20^0C. The leaf sap was extracted using freeze-thaw method (Tomos *et al.* 1984) and osmolality was measured using the vapour pressure osmometer

(Vapro, Wescor Inc. Logan, Utah, USA). The sample size for both control and salt treated plants was n=6. Measured Na^+ and K^+ concentrations were then used to calculate their relative contribution towards overall sap osmotic potential. Contribution of Cl- was estimated as 1.2 of that for Na^+, as previously described in the literature (James et al., 2006; Puniran-Hartley et al., 2014).

5.2.9 Statistical analysis

The data was analysed using student's t-test method and the significant level of mean difference was compared at probability value of 0.05 (*), 0.01 (**) and 0.001 (***) using a statistical software SPSS version 22 (IBM support portal, USA).

5.3 Results

5.3.1 Generation of barley plants expressing *V-ATPase Subunit C*

Transgenic barley (cv. WI4330, kindly supplied by the University of Adelaide Barley Breeding Program) expressing the *V-ATPase subunit C* (*VHA-C*) was generated using an agrobacterium mediated transformation (Jacobs *et al.* 2007; Tingay *et al.* 1997). Four *AtVHA-C* expressing lines, designated as OE1-OE4 (*35S:AtVHA-C*-1 to *35S:AtVHA-C*-4), and one non-transformed WI4330 line (WT) were included in this study. The OE4 line did not show consistent phenotype throughout all experiments, most likely due to a detrimental effect from the transformation process, and was therefore omitted from analysis. The presence of the *VHA-C* gene in the genome of transgenic lines and its absence in the WT were confirmed using PCR on genomic DNA extracted from the leaf (Fig. 5.1a). The expression of the *VHA-C* gene in transgenic lines and the absence of its expression in the WT were confirmed by RT-PCR (Fig. 5.1b).

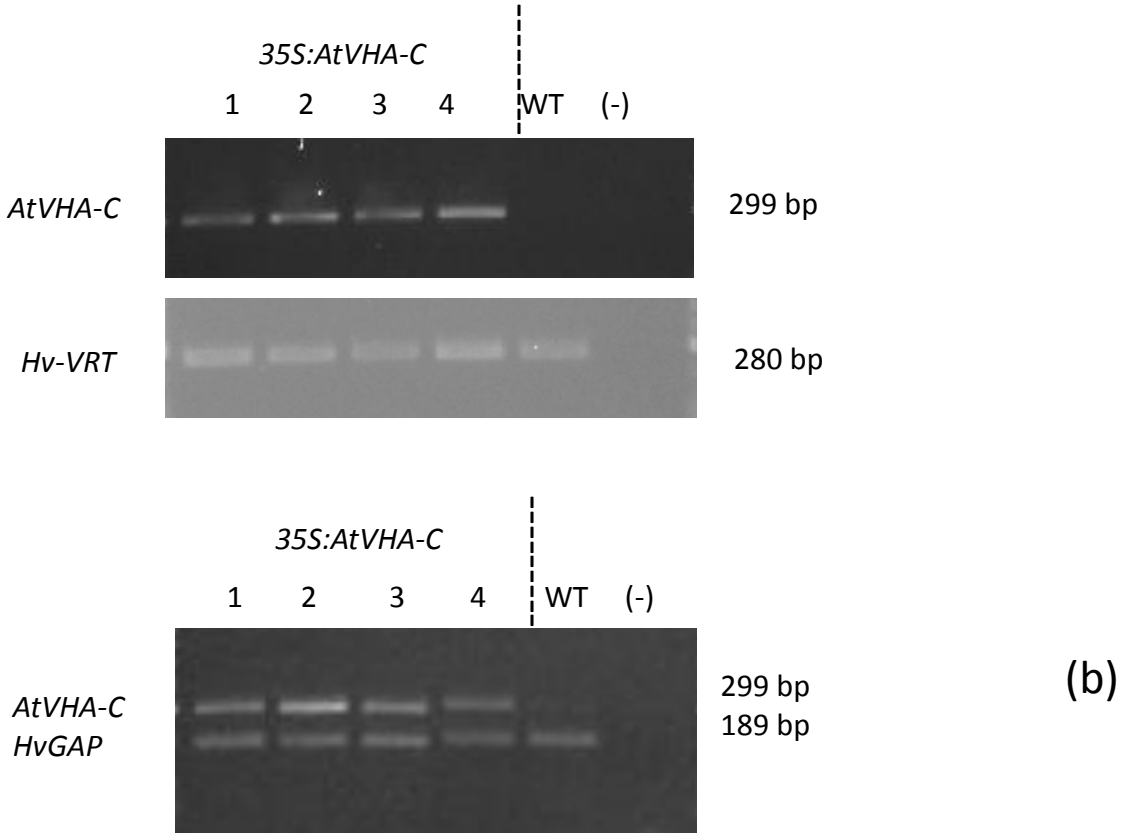

Figure 5.1 Genotyping *AtVHA-C* transgenic barley. (a) Detection of transgene presence by PCR using specific primers for *AtVHA-C* and *Hv-VRT2* (internal control); (b) Expression analysis of the *AtVHA-C* gene using RT-PCR with specific primers for the gene and *HvGAP* specific primers (internal control). Lanes *35S:AtVHA-C*-1, 2, 3, & 4 are transgenic *AtVHA-C* barley lines, WT (non-transformed barley cv. WI4330), (-) negative control (water).

5.3.2 Transgenic barley expressing *V-ATPase subunit C* has improved overall salinity stress tolerance and grain yield

The *AtVHA-C* expressing lines (OE) had larger seed size and were significantly ($P \leq 0.001$) heavier compared to WT barley plants after growing under 300 mM NaCl stress (Fig. 5.2; Table 5.2). The better performance of the OE lines was also reflected in plant phenotype (Fig. 5.3), with the transgenic lines producing more tillers and remaining green, while the WT plants showed signs of severe senescence. While no significant (at $P \leq 0.05$) difference was found between the dry weight (DW) of OE lines and WT under control conditions (0 mM NaCl; Fig. 5.4a), and an slight increase in the DW of transgenic plants under salt stressed conditions (300 mM NaCl; Fig 5.4b), the relative DW reduction under saline conditions was significantly lower in all transgenic lines compared with WT (Fig. 5.4c). The relative DW of salt-treated overexpressing lines ranged within 43 to 47% of control vs only 35% in WT (Fig. 5.4c). While there was little difference in plant height in the 0 mM treatment (Fig 5.4d), all saline-grown OE lines were much taller compared with WT (Fig. 4e; significant at $P \leq 0.001$), and showed much less height reduction compared with non-salt treated plants (Fig 5.4f; significant at $P \leq 0.05$).

The number of seeds per plant was not significantly (at $P \leq 0.05$) different between OE lines and WT neither under control (Fig. 5.5a) nor saline (Fig. 5.5b). However, the OE lines showed significant ($P \leq 0.05$) lower reduction in seed number when grown in 300mM NaCl (Fig 5.5c). While the grain yield per plant (g per plant) was not significantly (at $P \leq 0.05$) different between the OE lines and WT under 0 mM conditions (Fig. 5.5d) there was significantly higher grain yield in two of three OE lines under 300 mM (OE-1 and OE-2; significant at $p \leq 0.001$ and $p \leq 0.01$ respectively; Fig. 5.5e). This result in a greater maintenance of grain yield in the transgenic lines compared to the control (Fig 5.5f).

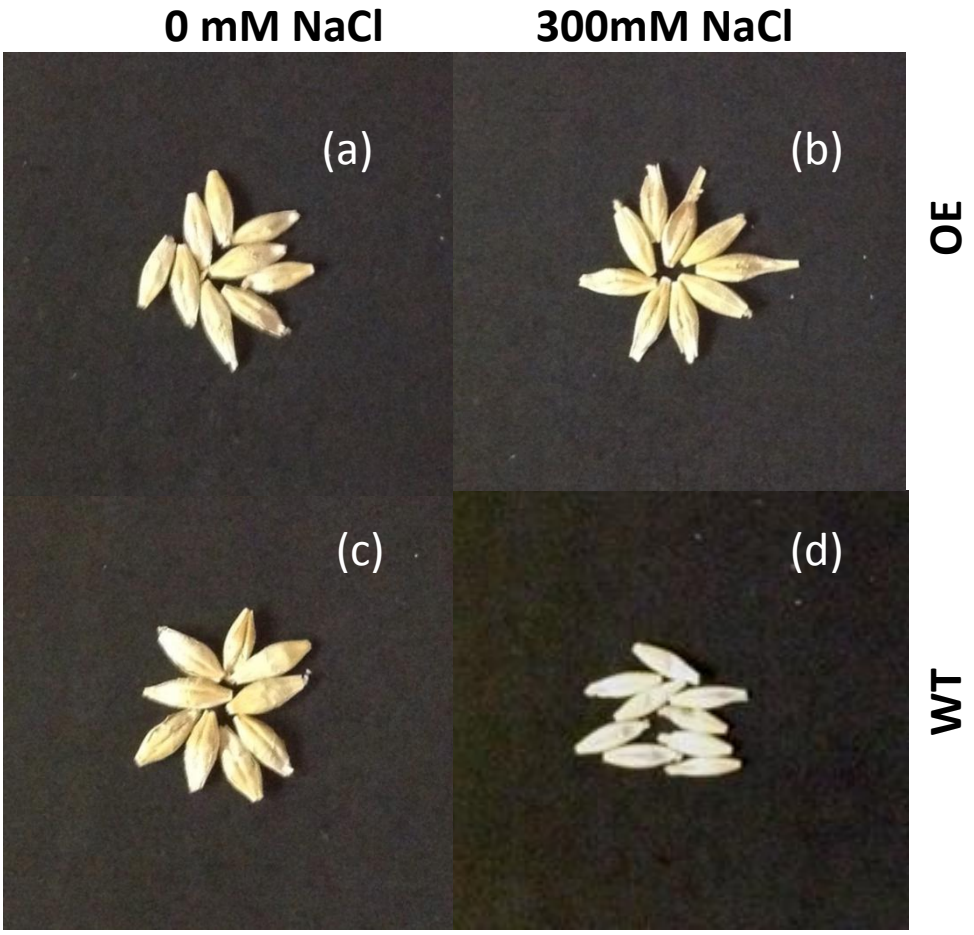

Figure 5.2 Seed size of transgenic lines. The difference in seed size is illustrated for one (OE3 line) of four *35S:AtVHA-C* overexpressing lines under no salt (a, c) and salt stress (b,d) conditions. The other lines show similar results.

Table 5.1 Seed weight of *AtVHA-C* expressing barley lines and wild type under 0 mM NaCl and 300 mM NaCl growth condition. *** Significant at $P \leq 0.001$. Results are the mean ± s.e. (n = 9)

Cultivars	**Seed Weight (mg)**
WT control	68.1 ± 4.0
OE control	58.0 ± 3.0
WT salt	15.0 ± 0.9
OE salt	48.1 ± 3.6***

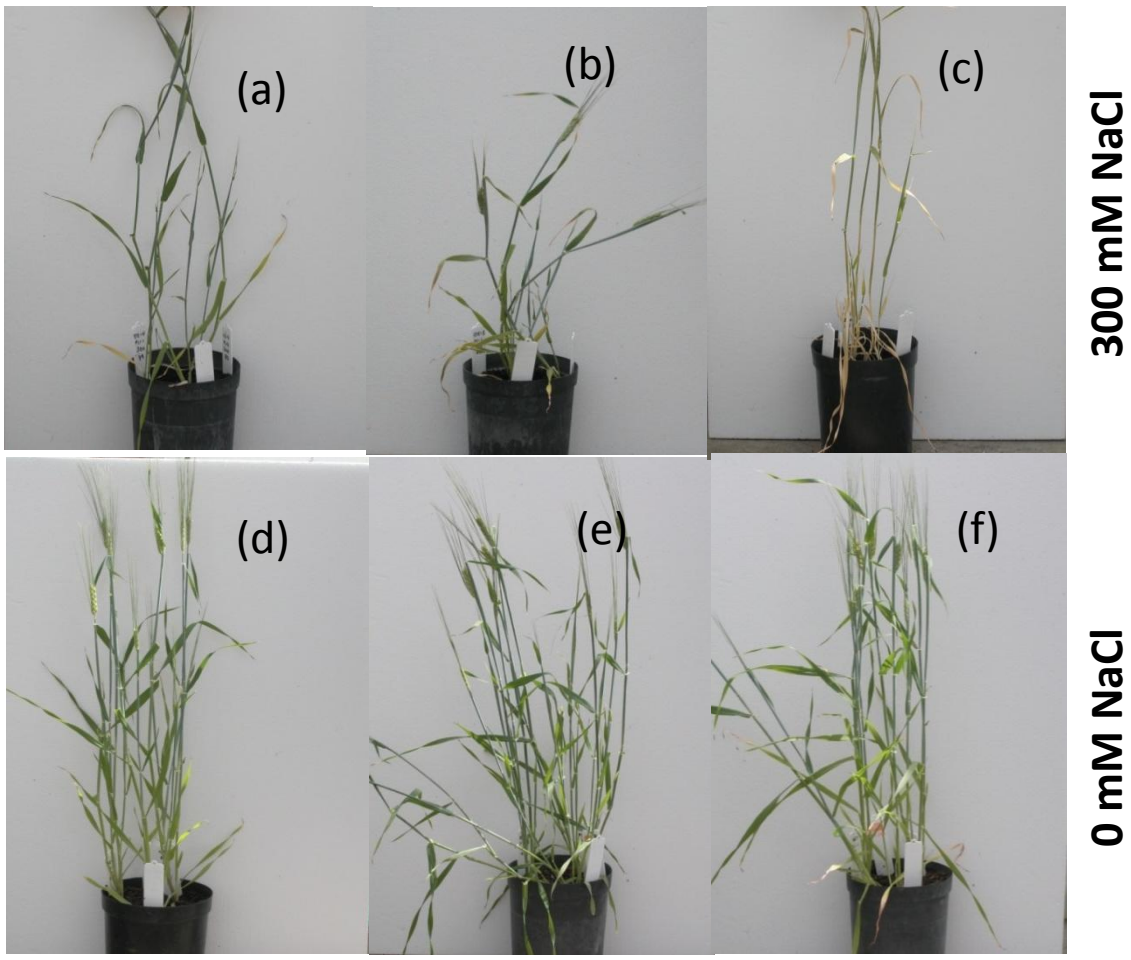

Figure 5.3 Phenotypic difference between non-transformed and overexpressing lines. For simplicity, only two (of four) OE lines are shown. (a-c) Plants treated with 300 mM NaCl for five weeks; (d-f) zero NaCl treatment (no salt stress). The photos were taken 8 weeks after sowing.

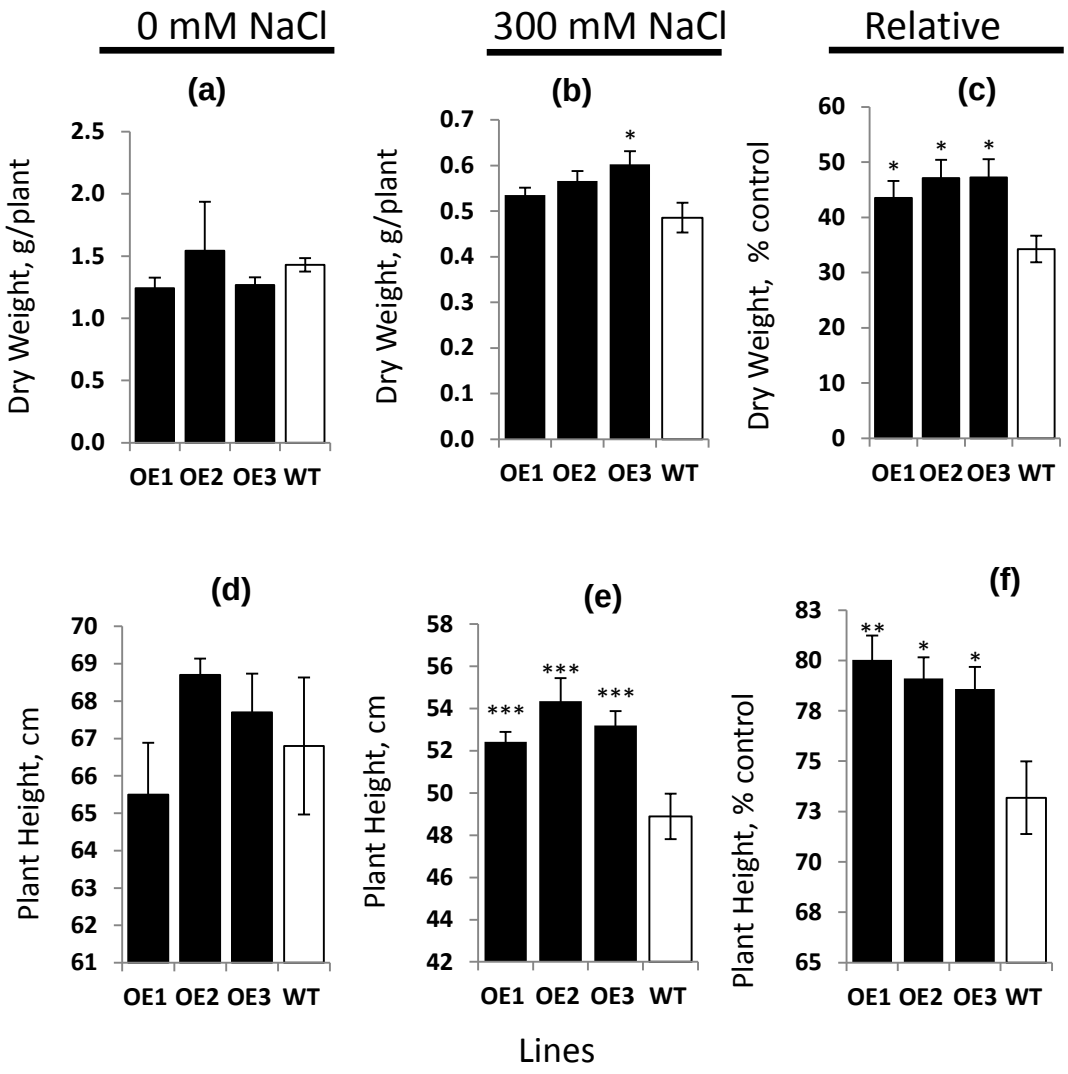

Figure 5.4 Comparative analysis of agronomic characteristics between non-transformed and OE plants. Shoot dry weight (a-c) and plant heights (d-f) are shown for plants grown under zero mM NaCl (no salt; (a, d)) and 300 mM NaCl (saline; b, e) conditions. Panels (c, f) report relative shoot dry weight and plant height, respectively (calculated for salt treated plants as % of control). Results are mean ± SE (n=6-15). Measurements are taken 12 weeks after sowing. Asterisks indicate the significant levels of the difference compared with wild type at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$.

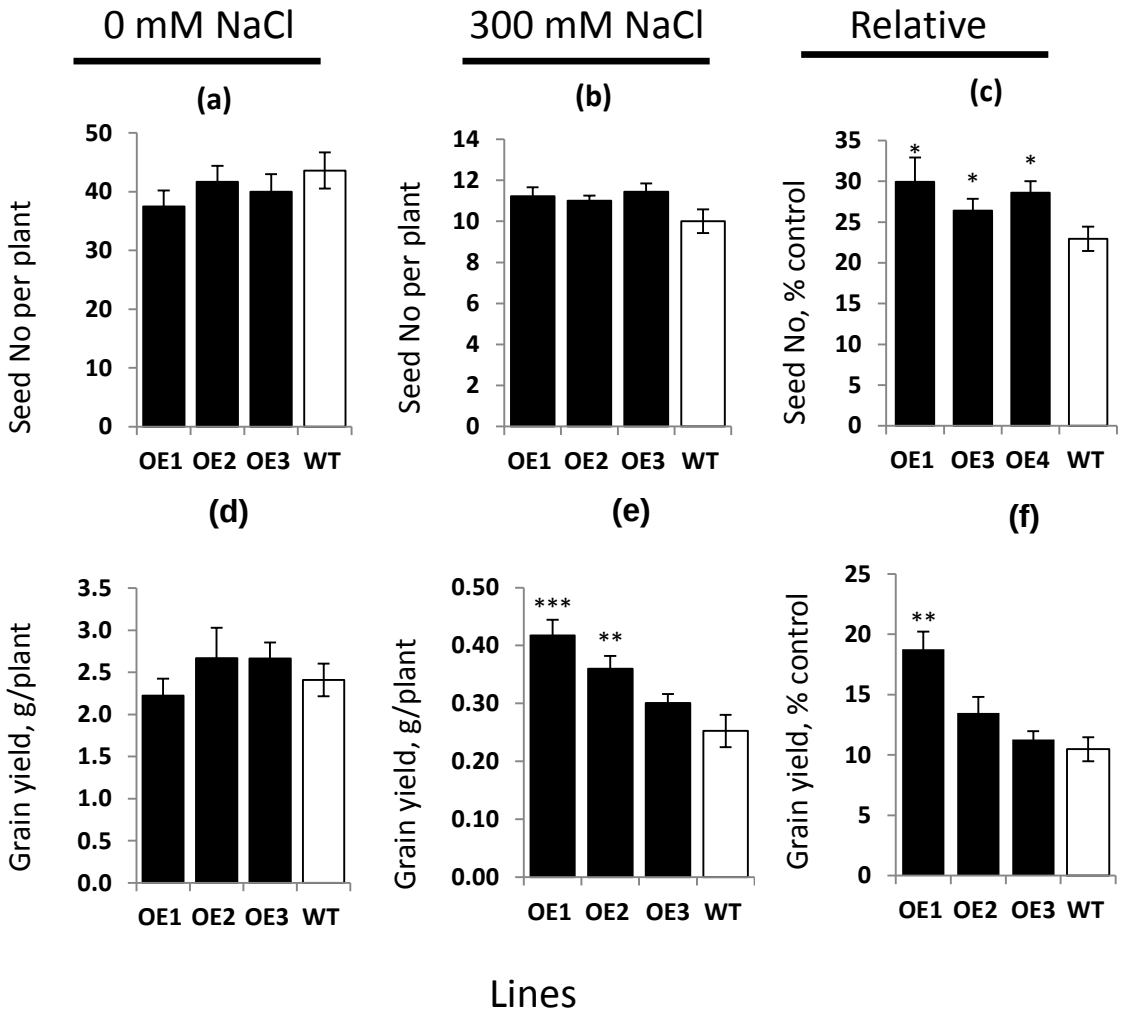

Figure 5.5 Seed number (a-c) and total grain yield (d-f) of OE lines as compared with non-transformed controls. (a, d) – 0 mM NaCl treatment; (b, e) – 300 mM NaCl treatment; (c, f) - relative values (calculated for salt-treated plants as % of control). Results are mean ± SE (n=6 to 15). Measurements are taken 12 weeks after sowing. Asterisks indicate the significant levels of the difference compared with wild type at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$.

There was no significant difference in the number of chlorotic leaves under salinity stress between salt-grown OE lines and WT (Fig. 5.6a) but all OE lines showed a significantly ($P \leq 0.001$) lower number of necrotic leaves compared with WT (Fig. 5.6b).

The stomatal conductance of OE lines ranged between 56 and 95 $mmolm^{-2}s^{-1}$ with the stomatal conductance of the OE lines significantly lower than the wild type in 0 mM NaCl (Fig. 5.7a). When the plants treated with salt (300 mM NaCl), all three OE lines had approximately 3-fold higher stomatal conductance than WT (17 to 19 $mmolm^{-2}s^{-1}$ vs only 6.1 ± 0.12 $mmolm^{-2}s^{-1}$ for WT; significant at $P \leq 0.001$; Fig. 5.7b). The chlorophyll content was not significantly different between OE lines and WT under control condition (Fig. 5.7d) but was reduced to a less extent under saline conditions (significant at $P \leq 0.05$; Fig. 5.7f).

5.3.3 Transgenic barley expressing *V-ATPase subunit C* has increased leaf Na^+ and K^+ and these ions contributed towards osmotic adjustment

The OE lines had significantly higher leaf Na^+ content than WT plants, both under 0 mM NaCl (Fig. 8a) and 300 mM NaCl (Fig. 5.8b) treatments ($P \leq 0.001$). However, the relative increase in leaf Na^+ under salt stress was less in OE lines compared to WT (six to 7 fold vs 9.2 fold in WT; significant at $P \leq 0.001$; Fig. 5.8c). Leaf K^+ content for all the OE lines and WT was not significantly (at $P \leq 0.05$) different under control conditions (Fig. 5.8d) but all the OE lines showed much higher leaf K^+ content compared with WT under saline conditions (significant at $P \leq 0.001$; Fig. 5.8e). Two out of three OE lines (OE-1 and OE-2) have significantly increased the overall leaf K^+ content (1.5 and 1.4 folds, respectively) while WT plants were not able to do this when grown under saline conditions (Fig. 5.8f).

Osmolality of leaf sap shows there are no differences between OE lines and WT (Table 5.1) but the measured contribution of inorganic ions in WT plants was lower than in OE lines. The relative contribution of inorganic and organic osmolytes towards osmotic adjustment in barley leaves were estimated from these results. In WT, the overall contribution of three

major inorganic osmolytes (Na^+, Cl^-, and K^+) was only ~34%, implying that the major bulk of osmotic adjustment under saline conditions (~66%, or two thirds) was achieved by other means, such as *de novo* synthesis of organic osmolytes (Table 5.1). In a stark contrast, contribution of organic osmolytes towards osmotic adjustment in OE lines varied between 49 and 52% (Table 5.1; significant compared with WT a $P \leq 0.01$), e.g. was by ~15% less than in WT.

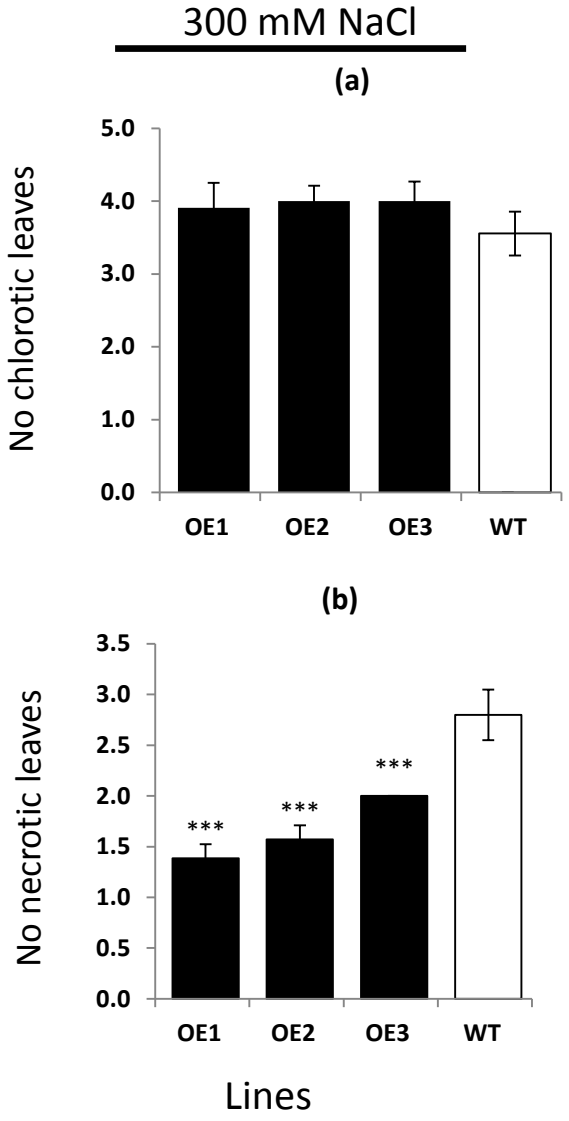

Figure 5.6 Difference in the number of chlorotic (a) and necrotic (b) leaves between OE lines and non-transformed barley grown in 300 mM NaCl. Results are mean ± SE (n=6 to 15). Measurement was taken 8 weeks after sowing. Asterisks indicate the significant levels of the difference compared with wild type at $^{***}P \leq 0.001$.

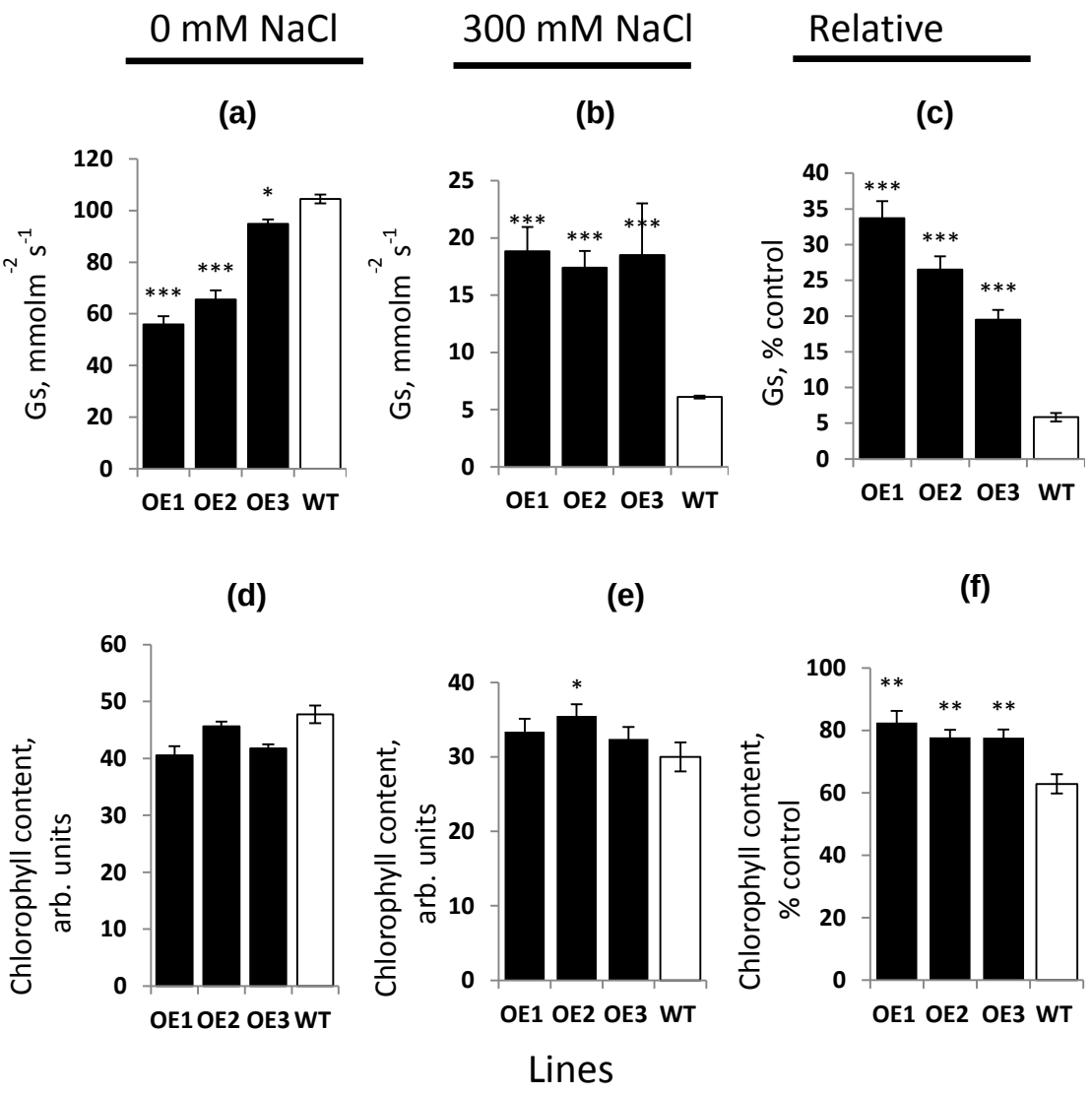

Figure 5.7 Stomatal conductance (a-c) and leaf chlorophyll content (SPAD values; (d-f)) of OE lines as compared with non-transformed barley. (a, d) – 0 mM NaCl treatment; (b, e) – 300 mM NaCl treatment; (c, f) - relative values (calculated for salt-treated plants as % of control). Results are mean ± SE (n=6 to 15). Measurement was taken 8 weeks after sowing. Asterisks indicate the significant levels of the difference compared with wild type at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$.

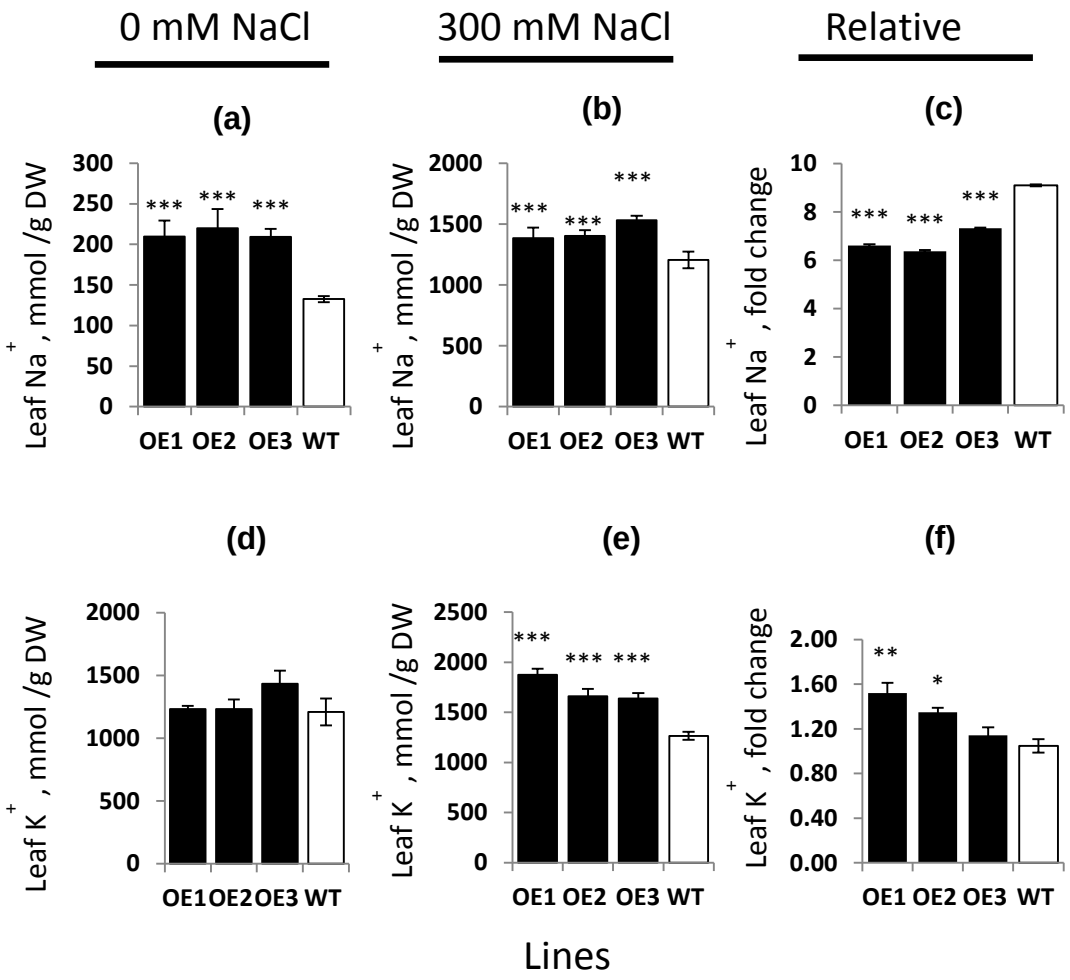

Figure 5.8 Leaf Na^+ (a-c) and K^+ (d-f) content of OE lines as compared with non-transformed barley. (a, d) – 0 mM NaCl treatment; (b, e) – 300 mM NaCl treatment; (c, f) - relative values (fold changes in salt-treated plants compared with control). Results are mean ± SE (n=6 to 15). Measurement was taken 8 weeks after sowing. Asterisks indicate the significant levels of the difference compared with wild type at $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$.

Table 5.2 Relative contribution of Na^+, K^+ and Cl^- towards total shoot sap osmolality in transgenic barley leaves grown under saline growth condition. Barley lines designated as *35S:AtVHA-C*-1 (OE-1), *35S:AtVHA-C*-2 (OE-2), *35S:AtVHA-C*-3 (OE-3) and non-transformed control (WT). * Significant compared with WT at $P \leq 0.01$.

	Measured		**Calculated**	**Relative Contribution**	
Lines	Total osmolality, mOsm	Contribution of inorganic ions, mOsm	Contribution of organic ions, mOsm	Organic, %	Inorganic,%
OE1	967	492	475.1	49.1	50.9**
OE2	1002	474	527.5	52.6	47.4**
OE3	986	500	485.7	49.3	50.7**
WT	1129	392	737.1	65.3	34.7

5.4 Discussion

5.4.1 Transgenic *VHA-C* barley improved growth and grain yield under saline growth condition

The activity of V-ATPase increased under salt stress (Silva *et al.* 2010), and both V-ATPase and *NHX* transcripts were up-regulated under salt stress (Qiu *et al.* 2007). Transgenic plants expressing subunits of V-ATPase have shown improvement in growth and better performance under saline condition in several species, such as the c1 subunit in rice (Baisakh *et al.* 2012) and tobacco (Xu *et al.* 2011), as well as a variety of V-ATPase subunits in Arabidopsis (He *et al.* 2014; Wang *et al.* 2011; Zhao *et al.* 2009). All the above examples were related to crops species classified as "salt excluders" which do not normally rely on vacuolar Na^+ sequestration mechanisms (Shabala 2013). For understanding of the growth and physiological response of the *AtVHA-C* gene in barley plants, the gene (*At1g12840*) was successfully cloned from Arabidopsis cDNA into a transformation vector, under the control of a the *CaMV 35S* promoter and transformed into barley using Agrobacterium mediated transformation. In this

study we show, for the first time, that expressing *VHA-C* from Arabidopsis also enhances salinity stress tolerance in a crop plant (barley) which has the ability to accumulate significant levels of shoot Na^+. .

The transgenic lines expressing *VHA-C* not only showed significant ($P<0.05$ and higher) dry weight and plant height when grown under saline conditions (Fig. 5.4) but also higher absolute grain yield (Fig 5.5e). As far as we are aware this is the first work showing transgenic plants expressing V-ATPase subunits have improvements in this important agronomical trait. The closest report we are aware of is improved yield in field grown barley plants expressing the H^+ V-PPase gene *AVP1* (Schilling *et al.* 2014).

5.4.2 Better stomata control contributes to enhanced salinity tolerance in transgenic plants

Previous reports suggest the beneficial effects of overexpressing V-H^+-ATPase transporters (subunits) were related to either improved plant water status, resulting from reduced stomata density and early stomata closure (Baisakh *et al.* 2012), or from increased activity of enzymatic antioxidants (AO) in transgenic lines (He *et al.* 2014). However, plant biomass (and, hence, dry weight) is directly proportional to its ability to assumilate CO_2 (which relies on gas exchange through open stomates) therefore reductions in stomata density and closure, as suggested by Baisakh et al, (2012), cannot be the sole reason *V-H^+-ATPase* expressing plants have greater salinty tolerance. In addition, there are many reports showing there is no direct correlation between AO activity and salinity stress tolerance in crops (Maksimovic *et al.* 2013). Many salt tolerant plants (such as halophytes) do not require high AO activity as they prevent formation of high levels of reactive oxygen species in the first place (Bose *et al.* 2014a). Therefore must be another mechanism(s) by which *V-H^+-ATPase* can improve salt tolerance in transgenic plants. From the data reported here, two physiological mechanisms were identified which would lead to improved performance of overexpression lines. The first

one was reduced number of necortic leaves (Fig 6b) and the second one – higher relative stomatal conductance (Fig 5.7c). The association between eexpressing V-ATPase subunits and stomatal opening and closing has been reported by (Allen *et al.* 2000) using *VHA-C* mutant (*det3*), and also by RNAi knockdown of *OsVHA-A* (Zhang *et al.* 2013). Two to three fold higher Gs values in transgenic lines compared with WT (Fig 5.7b) might account for better CO_2 assimilation, and fewer necrotic leaves increased the overall photosynthetic leaf surface in transgenic place. Both these factors could contribute to increase plant biomass and, ultimately, higher grain yield in OE lines.

5.4.3 Transgenic lines expressing *VHA-C* have better Na^+ sequestration

All the transgenic plants expressing *VHA-C* showed higher leaf Na^+ content than the WT when grown under saline condition (Fig. 5.8b). At the same time, the chlorophyll content was not affected (Fig 5.7), and the number of necrotic leaves was reduced in OE lines (Fig 5.6). Taken together, these facts point out at highly efficient vacuolar Na^+ sequestration in transgenic plants. Na^+/H^+ antiporters, such as members of the NHX family (Apse *et al.* 2007; Barkla *et al.* 1995; Blumwald *et al.* 1985; Gaxiola *et al.* 1999) energized by the proton motive force generated by proton pumps. The improvement in salinity tolerance in the *V-ATPase C* expressing barley may be due to an improvement in the H^+ gradient between the vacuole and the cytosol, so that native NHX transporters could compartmentalize more Na^+. Expressing the *subunit C* of the *V-ATPase*, therefore, could be instrumental to increase H^+-ATPase phosphorylation (Armbruster *et al.* 2004) or stability of its operation (Sze *et al.* 2002), therefore better energizing NHX activity and thus assisting Na^+ sequestration process. Consistent with this model, transgenic Arabidopsis expressing wheat *V-ATPase subunit E* showed increased Na^+ accumulation after 10 days of 120mM NaCl but with a lower Na^+ accumulation in the cytosol (Zhao *et al.* 2009). It remains to be seen whether the improved sequestration in the vacuole is due solely to an increase in the activity of Na^+/H^+ antiporter

which are already present in the cell or if there is also an up regulation in genes encoding Na^+/H^+ antiporters, resulting in a greater concentration (and more activity) of the transporters.

5.4.4 Plants expressing *VHA-C* showed higher K^+ retention

The barley plants expressing *VHA-C* showed significantly higher leaf K^+ content compared with WT (Fig. 5.8). This feature correlated with the fewer number of necrotic leaves in salt stressed plants (Fig 5.6). This suggests that K^+ retention in the shoot was essential for preventing salt stress-induced senescence, a programmed cell death process (PCD). The loss of cytosolic K^+ in cells under stress induces the activation of caspase-like proteases and endonucleases and, hence causes PCD in plants (Demidchik *et al.* 2010; Shabala *et al.* 2007) and mammalian cells (Hughes *et al.* 1998, 1999). Potassium retention in both shoots and roots are crucial mechanisms in salt tolerance in barley which was corroborated by K^+ kinetics study on shoots and roots of barley and on roots using electrophysiological studies (Adem *et al.* 2014). Recently, Wu *et al.*, (2013) and Wu *et al.*, (2014) showed K^+ retention in the leaf mesophyll is a contributing factor towards salt tolerance in barley, as measured by non-invasive ion flux. Importantly, a number of NHX transporters have been shown to have a higher affinity for K^+ than Na^+ (Barragan *et al.* 2012; Bassil *et al.* 2011b; Leidi *et al.* 2010; Rodriguez-Rosales *et al.* 2008) – enhanced proton pumping in *V-H^+ATPase C* expressing barley may therefore may enable higher accumulation of K^+ in cell vacuoles. This may contribute to increased cell turgor and better osmotic adjustment under saline conditions (as discussed below). Higher K^+ content in leaves may be also essential for more efficient stomata control, given the critical role of this nutrient it stomata movements (Anschütz *et al.* 2014).

5.4.5 Relative contribution of organic and inorganic osmolytes

Synthesis of organic osmolytes is energy expensive, particularly in stressed environments (Raven 1985), and plants with enhanced salt tolerance tend to use Na^+ as cheap osmoticum

for osmotic adjustment (Chen *et al.* 2007a). As shown in Table 5.1, WT plants relied predominantly (65.3% of the cell's osmotic potential) on organic osmolytes in its osmotic adjustment. *V-ATPase C* expressing lines, however, had a large proportion of inorganic osmolytes (specifically, Na^+ and K^+) to achieve ~ 50% of cell osmotic potential. The transgenic lines were therefore likely to expend less energy generating organic osmolytes and make better use of photosynthetic assimilate (Table 5.1). Osmotic adjustment using inorganic ions is predominant mechanisms in halophytes (Inan *et al.* 2004), and it appears here that some "halophytism" was highly beneficial for transgenic barley plants as well in our case.

Chapter 6 General Discussion

Soil salinity is taking nearly three hectares of arable land from conventional crop farming every minute (Shabala *et al.* 2014). Of the world's 5.2 billion ha land coverage, 3.6 billion is dryland. Dryland is impacted by erosion, soil degradation and salinization (Riadh *et al.* 2010). Half of the world's irrigated land is compromised by salinization, alkalization or waterlogging (Szabolcs 1994). In Australia, the clearing of vegetation for farmland that started a century ago has contributed to saline soil covering 2 million ha, with estimates that another 15 million ha are at risk of turning saline in the next 50 years (National Land and Water Resources Audit; http://audit.ea.gov.au). It is estimated that currently one-third of Australian soil and 67% of Australia's agricultural land has the potential for transient salinity (Rengasamy 2006).

The salinity problem can be tackled in a number of ways. One could involve changing farm management practices, such as efficient use of irrigation. For example, drip irrigation conserves water-use and hence reduces salinization. Meanwhile in rain-fed agriculture, rotation of annual crops with deep rooted perennial plants prevents the water table from rising thus reducing transfer of the salt to the surface. Salt tolerant plants are the most cost effective solution in combating salinization (Munns 2002). In broader term, the solution to the problem is two-fold. The first option is to intensify crop production using currently available arable land by improving the productivity of crops exposed to stress. This calls for a major leap in breeding crops for stress tolerance. This option is limited by the availability of genetic diversity, in other words, a smaller gene pool to be tapped into to develop stress tolerant crops (Colmer et al. 2005). The second option could be identification of genes from extremophiles and introducing these into traditional crops (Shabala 2013; Shabala et al. 2014).

Salinity tolerance in crops is a multifaceted physiological trait involving multiple mechanisms. The main ones are osmotic adjustment, minimizing Na^+ uptake by roots and/or increasing Na^+ efflux back to the soil, intracellular Na^+ sequestration, potassium retention in

the cytosol, tissue-specific Na^+ sequestration, control of xylem ion loading, excluding Na^+ from the shoot and oxidative stress tolerance (Munns *et al.* 2008; Shabala *et al.* 2012; Zhu 2003). This list of mechanisms can be grouped into three major clusters: (i) osmo-tolerance; (ii) sodium exclusion; and (iii) tissue tolerance mechanisms (Munns *et al.* 2008). In spite of the fact that significant progress has been made in explaining specific details of each of these mechanisms, the relative contribution of the afore-mentioned components to overall salinity tolerance remains unclear. Part of this thesis study, efforts were made to elucidate relative contribution of the mechanisms of salt tolerance in barley, with experiments performed that spanned from the cellular to the whole plant level.

To begin with, crop osmotolerance has been attributed to a plant's ability to increase *de novo* synthesis of compatible solutes (Chen *et al.* 2007a; Hasegawa *et al.* 2000; Munns *et al.* 2015; Shabala *et al.* 2011b). This has been a less efficient way towards salt tolerance, as it involves energy expensive ATP to synthesise osmolytes (Chen *et al.* 2007a). At least for salt tolerant crops like barley, the usage of osmolytes for osmotic adjustment is less likely to be the primary mechanism due to the energy cost instead Na^+ is preferentially used as a "cheap" osmoticum. The other major component of salinity tolerance is Na^+ exclusion. This mechanism employs the plasma membrane Na^+/H^+ exchanger SOS1, which is activated through a Ca^{2+} dependent signaling pathway via phosphorylation by interacting proteins SOS2 and SOS3 (Shi *et al.* 2003; Wu *et al.* 1996). This process energized by the plasma membrane H^+-ATPase (Palmgren *et al.* 2011). The Na^+ extruded to the medium by this mechanism however can enhance osmotic and ionic imbalance that exacerbates the existing salinity stress. This mechanism can only be used as a short-term solution and may not confer salinity tolerance under field conditions. The other mechanisms restrict Na^+ accumulation in shoots involve reducing Na^+ loading into the xylem (Munns *et al.* 2008; Tester *et al.* 2003) and increasing Na^+ retrieval from the xylem (Davenport *et al.* 2007). Recently, Munns *et al.*,

(2012) reported 25% grain yield increases in durum wheat due to the presence of *TmHKT1;5-A* introgressed into commercial durum wheat cultivar. The gene is responsible for retrieving Na^+ from the xylem. The other mechanism involves sodium retrieval from the shoot via recirculation to the root via phloem (Berthomieu *et al.* 2003). This mechanism is believed to use the sodium HKT transporter (Garciadeblas *et al.* 2003). Nevertheless, its debated that excluding Na^+ from the xylem may not always be plausible mechanism (Shabala *et al.* 2010). It's because some species that employ high tissue tolerance mechanism use Na^+ as a cheap osmoticum. In light of this, *HvHKT2;1* over-expressing barley plants showed higher xylem and leaf Na^+ content in saline conditions and showed increased salt tolerance (Mian *et al.* 2011). Our findings also provide a physiological evidence that higher shoot sodium correlates with tolerance in barley (Adem *et al.* 2014). This is explained as the ability of the plant to accumulate large amounts of sodium in the tissue without causing harm to cellular functions. This could be achieved by sequestering excess Na^+ away from the cytosol into the vacuole or away from the actively photosynthesizing tissue. This is effected by the tonoplast Na^+/H^+ exchanger (Apse *et al.* 1999; Blumwald 2000; Gaxiola *et al.* 1999) energized by vacuolar H^+-ATPase and vacuolar pyrophosphatase (Fukuda *et al.* 2004a).

Tissue tolerance is one of the various mechanisms used by plants for salt tolerance and is a major contributor to salt tolerance in barley. This tolerance mechanism involves sodium sequestration away from the cytosol where sodium is deleterious for normal cellular metabolism. Sodium ions are the main contributor to the disturbance of cell metabolism, enzymatic activity and ionic homeostasis (Ahmad *et al.* 2014; Anschütz *et al.* 2014; Shabala *et al.* 2014). For a plant to grow under saline conditions, sodium should be excluded from the shoot keeping the K^+ at high levels thereby maintaining a high K^+/Na^+ ratio, particularly in the leaves (Ren *et al.* 2005; Serrano *et al.* 2001; Shi *et al.* 2002a). To achieve the maintenance of high K^+/Na^+ ratios in the cytosol, plants extrude Na^+ out of the cell via the plasma

membrane and/or sequestering Na^+ in vacuoles. The compartmentalization process is vital for preventing Na^+ toxicity in the cytosol and for osmotic adjustment of a cell conferring salt tolerance (Blumwald *et al.* 2000). Na^+ is transported across the tonoplast into the vacuole by exchanging protons. A gene encoding the Na^+/H^+ exchanger *NHX1* was found to compartmentalize Na^+ in prevacuolar and vacuolar compartments (Nass *et al.* 1997; Nass *et al.* 1998). The NHX protein is located in the tonoplast and energized by Vacuolar H^+- ATPase and Vacuolar H^+-inorganic pyrophosphatase (Gaxiola *et al.* 2007; Sze *et al.* 1999). Expressing *AtNHX1* gene was reported to confer salt tolerance in salt sensitive categories of plants (Apse *et al.* 1999; Bayat *et al.* 2011; Wu *et al.* 2009; Wu *et al.* 2004; Xue *et al.* 2004; Zhang *et al.* 2001a; Zhang *et al.* 2001b). Recently, a new role has emerged for the NHX1 protein in that it also transports K^+ ions (Barragan *et al.* 2012; Bassil *et al.* 2011b). Despite the above literature claims, in this PhD study expressing *AtNHX1* gene in barley did not confer any additional salt tolerance. There are several possible explanations for why this is the case. One reason could be a misfolding of AtNHX1 protein rendering it inactive. The second reason could be due to mistargeting of the protein into other membranes. A similar phenomenon was observed in the over-expression of *AVP1* which was mistargeted to the plasma membrane (Gaxiola *et al.* 2012; Li *et al.* 2005). The third reason might involve lack of required post-translational modification such as phosphorylation. Kinases, such as SOS2, are important regulatory components in other salt tolerance signaling systems (Neuhaus *et al.* 2014; Weinl *et al.* 2009). A more physiological oriented reason may comprise back leak of Na^+ from the vacuole to the cytosol via Na^+ permeable SV (slow activating) and FV (fast activating) channels, which typically confer salt tolerance in halophyte plant (Bonales-Alatorre *et al.* 2013b; Hedrich *et al.* 1987; Shabala 2013). Another physiological reason is the importance on needing to have highly active V-ATPase (Krebs *et al.* 2010; Vera-Estrella *et al.* 1999; Wang *et al.* 2001) and V-PPiase (Guo *et al.* 2006; Parks *et al.* 2002; Vera-Estrella *et al.* 2005) necessary to support the

energy needs of the Na^+ sequestration process by NHX ((Shabala 2013). In the transgenic barley plants activities of these enzymes may have been insufficient to support enhanced Na^+ sequestration (Adem *et al.* 2015). Furthermore, a reduced K^+ pool in the cytosol resulting from its enhanced sequestration in the vacuole might in turn require osmolyte production to prevent dehydration, a process which is energetically costly and thus would lead to yield penalties. However, the compensation for the cytosolic K^+ loss by osmolyte production is not the case for tolerant barley genotypes (Chen *et al.* 2007a) and the genetic background of the barley plant used in this study was a comparatively tolerant cultivar (Adem *et al.* 2014). Sequestration of Na^+ in the vacuole is possible by Na^+ loading, energization of loading and retention (Shabala 2013). In light of this, it was shown that better salt tolerance was obtained by co-expression of the *AVP* with *NHX* (Zhao *et al.* 2006). Hence, studying the individual components for sequestration and pyramiding them would be highly advantageous. Therefore, studies are showing that over-expression of *AVP*1 improve tolerance to salinity and drought in Arabidopsis (Gaxiola *et al.* 2002; Gaxiola *et al.* 2001; Gaxiola *et al.* 2012; Guo *et al.* 2006). Similarly, the over-expression of VHA subunits has shown salt tolerant phenotypes. For instance, V-ATPase c1 subunit from halophyte grass improved rice growth under saline conditions (Baisakh *et al.* 2012). He *et al.*, (2014) and Wang *et al.*, (2011) reported the improvement of salt tolerance by cloning wheat V-ATPase subunit B in Arabidopsis. Furthermore, Zhao *et al.*, (2009) has shown salt tolerant plants by over-expressing V-ATPase subunit E in Arabidopsis. Similarly, salt tolerant plants were obtained by over-expressing V-ATPase subunit c (Xu *et al.* 2011). In our study, we have shown the improvement of biomass and grain yield of barley plants by expressing *Arabidopsis thaliana* V-ATPase subunit C (*AtVHA*-C). The improvement in grain yield and biomass in our V-ATPase transgenic lines was as a result of better osmotic adjustment, as shown by better accumulation of Na^+ and K^+ in the shoot and higher contribution of inorganic ions (Na^+ and Cl^-) to osmotic adjustment

than organic solutes. In this PhD study, it was shown there was a lack of phenotype when *AtNHX1* was expressed on its own whereas a salt tolerance phenotype was observed when *AtVHA-C* was expressed. Given that co-expression of *NHX* gene with genes that encode proton pumps have been shown to have the best phenotypes (Zhao *et al.* 2006), the two transgenic genotypes used in this study could be crossed to pyramid both the *AtNHX1* and *AtVHA-C* and the subsequent offspring tested to see if pyramiding these genes might result in a better salt tolerant barley plant than simply expressing *AtVHA-C* alone. Such a combination may also help to investigate the reason behind why no phenotype was seen in *AtNHX1* expressing plants. It might answer the questions as to whether there was not enough protons being pumped into the vacuole in the *NHX1* expressing barley for the NHX to work. The *AtVHA-C* and the combined genotype expressing both *AtNHX1* and *AtVHA-C* need to be tested in the field in multiple sites and for multiple years to see if they show grain yield improvement. The *AtVHA-C* barley plants and the pyramided barley plants containing these two genes could be used for farmers if they show consistent results in future field trials or they can be used to show the importance of these two genes in yield improvement in saline soils and thus representing a stepping stone to more non-transgenic options. This could be achieving by screening for the best barley germplasm that has good expression of the native *VHA-C* gene and develop a marker to the gene so it can be introgressed into an elite barley cultivar using conventional breeding method. An alternative strategy would be to use the CRISPR (clustered regularly interspaced short palindromic repeats)- Cas system which can be used to selectively modify or edits a target region (Cheng *et al.* 2013; Sander *et al.* 2014; Shan *et al.* 2013). In the case here this would involve modifying the promoter region of the *VHA-C* gene to constitutively express the gene without requiring the development of a transgenic barley plant. The CRISPR-Cas system is a recent invention and there is still an ongoing debate about whether crops developed using this system are GMOs or non-GMOs. It

is a potentially superior option to rapidly obtain and transfer plants with desired traits to breeders and producers.

Vacuolar Na^+ sequestration is not the only mechanism contributing to tissue tolerance. Potassium retention is also emerging as an important tissue tolerance mechanism in root (Chen *et al.* 2005; Chen *et al.* 2007b; Chen *et al.* 2008; Chen *et al.* 2007d) and leave tissues (Wu *et al.* 2013). The maintenance of high K^+ in the cytosol suppresses the activity of caspase like proteolytic and endonucleolytic enzymes triggering programmed cell death in salt affected cells (Demidchik *et al.* 2010; Shabala *et al.* 2007). Cytoloslic K^+ plays roles in assisting metabolic processes, such as protein synthesis, by enabling the binding of tRNA to ribosomes (Wyn Jones *et al.* 1979).

An additional mechanism of that contributes to salinity tolerance under tissue tolerance is oxidative stress tolerance. Studies have shown that significant amounts of ROS are generated in salt affected root and leaf tissues (Miller *et al.* 2008; Mittler 2002). The causal link between salinity and oxidative stress signaling and reactive species detoxification has emerged (Bose *et al.* 2014a). Our findings have shown improved prevention of hydroxyl radical- induced K^+ lose in tolerant barley cultivars. This can be an indication of cross protection between stress responses in this case oxidative stress with salinity stress. This finding is also consistent with the report on root response to H_2O_2 (Maksimovic *et al.* 2013). NADPH oxidase is responsible for the ROS generation under salinity stress (Sagi *et al.* 2006). Accordingly, we studied the barley homologue of *RBoHF* gene transcript level and showed the least down regulation in the leaves of salt sensitive cultivar, which suggests a causal link between NADPH oxidase activity and tissue tolerance. Unlike in the leaves, the root transcript level compared to the control was non-significant (Adem *et al.* 2014). In our study, the transcript level of barley homologue *NHX*, *RBoHF*, *AHA* and *GORK* genes that are responsible for Na^+/H^+ transport to the vacuole, NADPH oxidase (ROS generation), plasma membrane H^+ pumping and outward

rectified K^+ efflux, respectively were studied at one point in time at seedling stage in both roots and leaves. At the tested time point and growth stage, the transcript level was found not to be a good indicator of salinity tolerance. This result may be interpreted in a number of ways. First, salt tolerance genes were shown to fluctuate between days (e.g *HVP10*) (Shavrukov *et al.* 2013). Second, the transcript amount may not always have a predictive value towards salinity tolerance at the whole plant level or a cellular level, since the gene product could be dependent upon post translational modification such as phosphorylation and proper protein folding. Taken together, the above mentioned genes need to be studied at different time scale and in different growth stage of the shoot and in different region of the root to better understand their roles in plant salinity tolerance.

To recap, soil salinity is a major abiotic stress costing billions of dollars to the agricultural industry and to combat this stress, a number of strategies can be devised, however, the cost effective method is to improve crop salt tolerance through genetic manipulation. Improvement of salt tolerance in barley should focus on tissue tolerance mechanisms. Sodium sequestration is one of the tissue tolerance mechanism and this study has indicated that different approaches must be used in improving salt tolerance where the NHX expression shown to not benefiting barley plants in improving salt tolerance unlike salt excluder crops. In light of this, the future efforts in sodium sequestration especially in a salt including crop like barley ought to consider co-expression of other genes involved in the sequestration process. Results have also shown that genes with specific function like NHX need the help of other genes such as pumps and those involved in Na^+ leaks. However, improvements in halotolerance using genes with general functions, for instance, proton pumps, as shown by expressing *AtVHA-C* (our study) and *AVP* in barley (Schilling *et al.* 2014), has shown to improve salt tolerance when expressed as single gene. Hence, the nature of the gene to be

expressed must be looked into first before we express it as single gene or co-express it with other genes.

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Appendix

Cloning of three genes was performed namely Arabidopsis V-ATPase subunit C and subunit D. Mostly, the general steps for developing transgenic lines that the salt group in ACPFG follows are: (1) identifying the Gene of Interest (GOI); (2) searching the gene from the sequence database; (3) designing primers; (4) optimizing PCR condition to amplify the GOI; (5) Inserting the GOI into entry clone-the one used is TOPO gateway entry clone; (6) checking the insert is in correct orientation by restriction digestion; (7) checking the sequence integrity-to avoid SNPs; (8) transferring the GOI to destination Vector (pMDC32) and finally, (9) submitting the GOI in the destination vector for transformation. In addition to this, characterization of Arabidopsis *NHX1* and V-ATPase subunit C genes in barley were made.

Generation of overexpression barley lines of V-ATPase subunit C&D

The Gene sequences were obtained from databases (e.g NCBI) and primers from the cDNA of these sequences were designed. These candidate genes were amplified using PCR and to get a clear band, the PCR condition has to be optimized. Fortunately, it was possible to amplify the V-ATPase subunit C&D using non proof reading DNA polymerase, as using a proof reading, high fidelity polymerase is expensive during optimization step.

After obtaining a clear band of the two V-ATPase genes using normal DNA polymerase, these same genes were amplified using a high fidelity and proof reading capable DNA polymerase called Elongase enzyme mix. The expected band size for subunit D is **786bp** and for subunit **C 1128bp**. The bands are presented in the figure below (Fig 1). The DNA ladder used was 100bp. Half of the volume of these PCR products was loaded on to gel and the remaining half purified by PCR purification kit and used further in the cloning process.

The PCR condition to get the bands shown below was as follows:

Components	Volume/reaction
10mM dNTP mix	0.5µL
5X Buffer A	2.5 µL
5X Buffer B	2.5 µL
Elongase Enzyme mix	0.5µL
MilliQ Water	16.5 µL
	22.5µL
To the Tubes	
Template (root At cDNA)	0.5µL
Forward Primer (10µM)	1 µL
Reverse Primer (10µM)	1µL
	25µL

The thermocycler was programmed as initial denaturation at 94^0C for 30 seconds, denaturation at 94^0C for 30 seconds, annealing at 55^0C for 30 seconds and extension at 68^0C for 60 seconds. The cycle of denaturation to extension was repeated for 35 cycles.

Figure 1. The amplified PCR product of At V-ATPase subunit C and D

As mentioned above, the remaining purified PCR products of the two genes were cloned into pCR8/GW/TOPO TA Gateway @ entry clone (entry vector in short). For doing so, the

following procedure was used. 4 µL of purified PCR product, 1 µL salt solution, 0.5 µL pCR8/GW/TOPO TA Gateway @ entry clone and 0.5 µL of milliQ water were gently mixed and incubated at room temperature for 3 hours covered in aluminium foil. This procedure will allow the GOI to be inserted into entry clone. The next step was to transform the entry clone into competent bacteria cells.

For heat shock transformation, 20 µL TOPO competent cells was thawed on ice for 10-15 minutes and 2 µL of the entry vector containing GOI (pCR8) was added into the competent cells. These mixtures were heat shock for 90 seconds at 42 ^{0}C and immediately put back on ice for 5 minutes and 800µL of SOB solution (to recover the cells) were added in the laminar flow. The above mixture was incubated at 37^{0}C for 1 hour shaking. After 1 hour, the transformed cells were plated onto a petri-plate containing LB agar with spectinomyces for growing only the transformed cells. The petri –plates were incubated at 37^{0}C overnight.

From the Petri plates, single colonies were picked by new tips and inoculated LB media and cultured overnight at 37^{0}C in the incubator-shaker. Finally, Plasmid DNA was extracted from the cultured bacteria cells using Plasmid mini prep kit.

To check the insertion of the gene in the vector, which might be optional to do, restriction digestion using EcoRI was made. Among the 12 colonies of V-ATPase subunit D (designated here as D) and subunit C (designated as C), 11 showed insertion for D and 6 for C, which are D1,D2,D3,D4,D5,D6,D7,D9,D10,D11,D12 & C1,C2,C3,C4,C6,C7 (Fig 2).

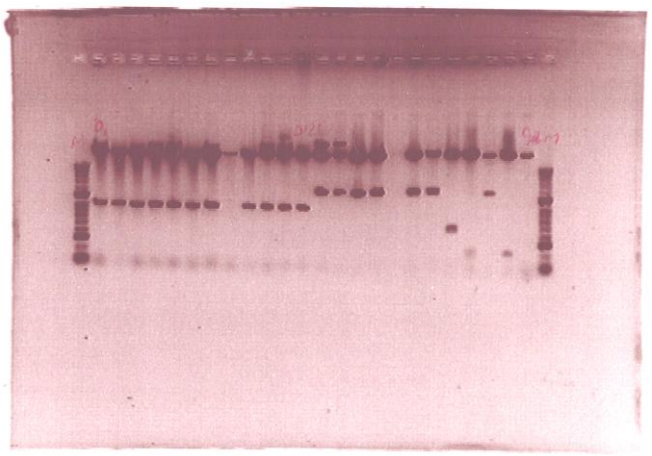
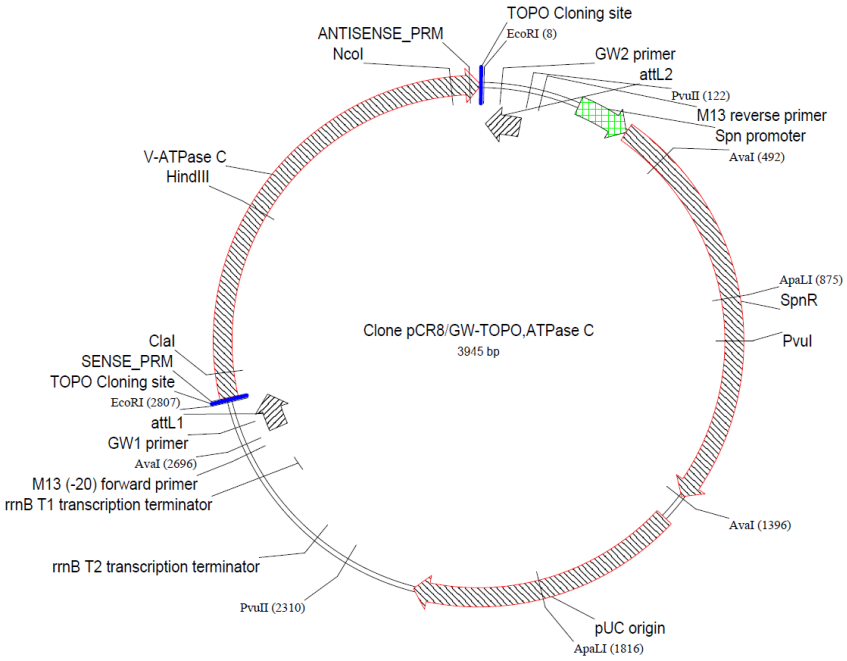


Figure 2. EcoRI digestion to see the presence of the GOI in our clones



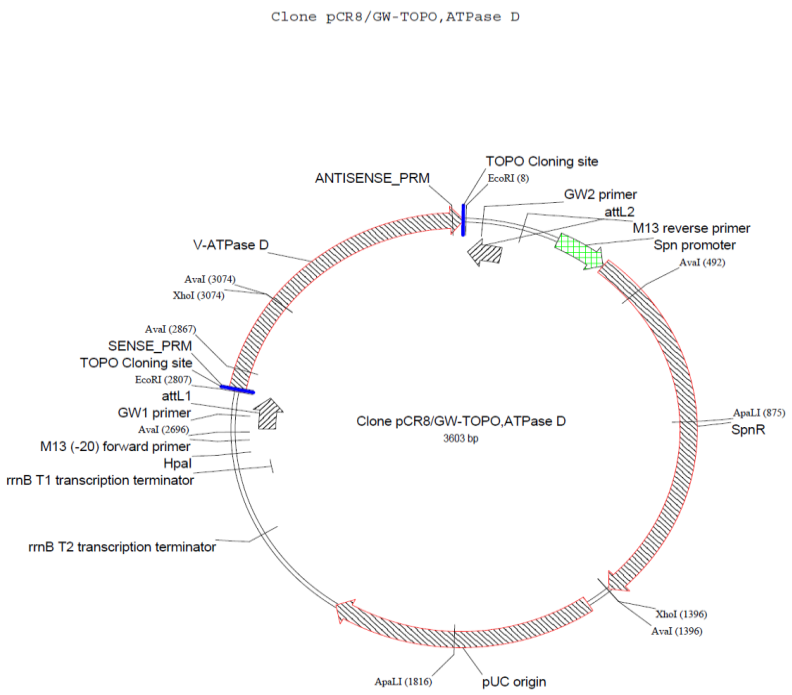


Figure 3. The construct map of VATPase subunit C & D in the entry clone.

Checking the correct orientation of the GOI in the entry clone

For checking the correct orientation of the GOI in the entry clone, the clone was double digested with two restriction enzymes. HpaI and XhoI for VATPase subunit D gene which gives an expected band size of 1925bp, 1241bp, and 437bp. And, ClaI and PvuII was used for V-ATPase subunit C gene that is expected to give band size of 2188bp, 1186bp, and 571bp.

Restriction digestion conditions for th VATPase subunit D gene insert

Components	Volume/reaction
10X NEBuffer	1.0µL
Hpa I	0.2 µL
Xho I	0.2 µL
BSA (100X)	0.1µL
MilliQ Water	6.5 µL
	8.0µL
To the Tubes	
Plasmid DNA	2.0µL
	10µL

Restriction digestion conditions for th VATPase subunit C gene insert

Components	Volume/reaction
10X NEBuffer	1.0µL
Cla I	0.2 µL
Pvu II	0.2 µL
BSA (100X)	0.1µL
MilliQ Water	6.5 µL
	8.0µL
To the Tubes	
Plasmid DNA	2.0µL
	10µL

Firstly, out of the 12 clones for each of the genes, 4 clones from each gene were double digested to see whether the gene is correctly oriented in the clone and it was found out that 3 of the clones for the VATPase D gene (D1, D2 and D3) were inserted in the correct orientation. However, only one clone for the VATPase C gene were found to have correct orientation of the insert. The DNA ladder used was 1kb and the clones with correct orientation are marked in bold at the gel picture below (Fig.4).

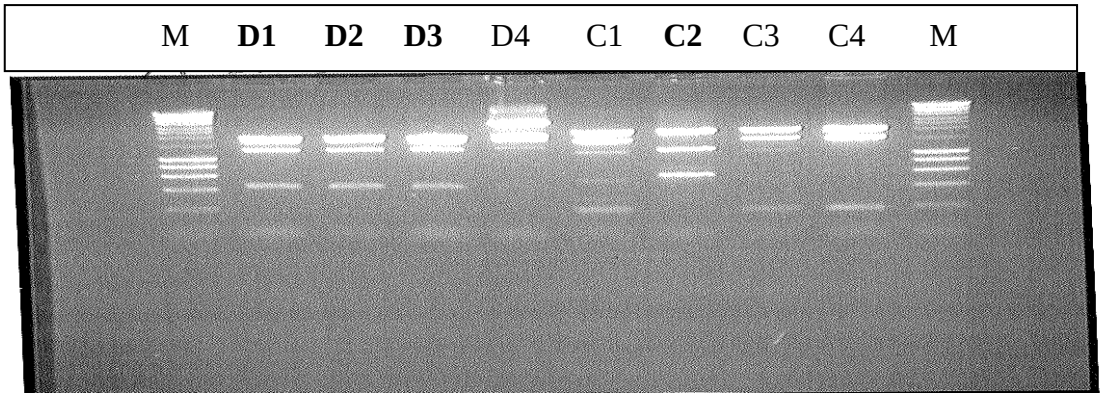


Figure 4. Four clones from each of the D and C genes double digested with restriction enzyme.

The next step, after checking the correct orientation of the gene in the entry clone, is to sequence the gene for examining the integrity of the sequence, i.e., the presence of Single Nucleotide Polymorphisms (SNPs) in the sequence that tend to happen due to the errors introduced to the sequence in all the cloning steps involved. Usually, it is good to sequence more than one clone to widen the chance of getting clones with no SNPs. Nevertheless, in our result explained above, only one clone for the C gene showed correct orientation of the GOI. Hence, additional clones for the C gene have to be tested for correct orientation of the GOI to have at least two clones with correct orientation of the GOI which can be subjected to sequencing. Accordingly, all the available clones of the C gene which had the GOI in them were double digested with their respective restriction enzymes. Among the tested clones, only

one was found to have the GOI in the correct orientation which was the C7 clone. This makes the clones from the C gene two that are ready for sequencing. The C7 clone that showed the correct orientation of the C gene is marked in bold in the gel below (Fig. 5).

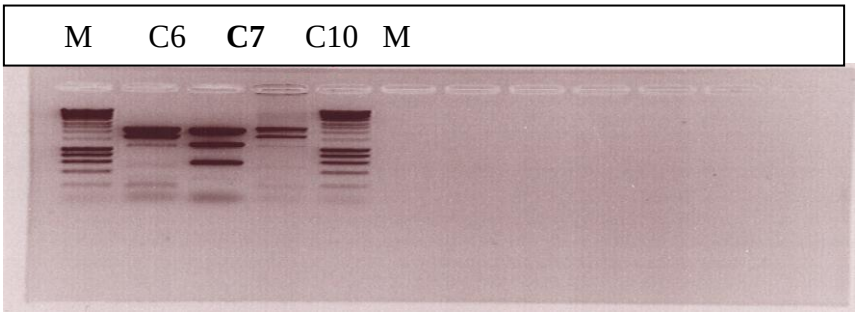


Figure 5. Three clones from the C gene double digested with restriction enzyme.

Sequencing to check the presence of SNPs

As described above, it is good to sequence more than one clone and hence, two clones from each gene were sequenced using sense primer GW1 and antisense primer GW2. The sequencing reactions were made three times for each primer per clone which makes it six reactions per clone and a total of 12 per gene and 24 for both genes. The sequencing reaction used the following reagents and PCR conditions.

Components	Volume/reaction
Big Dye Terminator V.3.1	1.0µL
5X Big Dye Terminator dilution buffer	3.5 µL
MilliQ Water	4.68 µL
	9.18µL
To the Tubes	
Plasmid DNA	0.5µL
Primer (GW1 or GW2)	0.32 µL
	10µL

The PCR condition was initial denaturation at 96^0C for 2 minutes, denaturation at 96^0C for 10 seconds, annealing at 50^0C for 5 seconds, extension at 60^0C for 4 minutes and the cycle is repeated 30 times. Then, to clean up the sequencing reaction, it was allowed to get to room temperature and transferred to 1.5 ml eppendorf tubes. 75 µL of 0.2 mM MgSO4 in 70 % ethanol was added, mixed by vortexing and allowed to sit at room temperature for 15 minutes. The solution was centrifuged for 15 minutes at 15,000g and the supernatant was removed with a pipette. 75 µL of 70% ethanol was added and centrifuged for 15 minutes at 15,000g. The supernatant removed and the pellet air dried by protecting it from light as long as it dries and submitted for sequencing.

The sequencing result showed that the D gene had SNPs and therefore, the whole process of cloning has to be repeated for this gene. Fortunately, for the C gene, the sequences made using GW1 primers were good except some poor sequencing reads for the GW2 sequences. Hence, the C sequence was repeated only using GW2 primer along with other clones of D gene. Finally, the sequence of the C gene showed no SNPs for the sequence reads in both the sense (GW1) and antisense (GW2) primers that makes it ready to go to the next steps of cloning ,i.e., cloning into the destination vector using LR reaction. The LR reaction components and the volume used and the incubation condition were as follows:

Components	Volume in µL
VATPase C in PCR8	4
pMDC32 (destination vector)	1
5X LR enzyme mix	2
TE Buffer (pH 8)	3
	10

The above solution was incubated at 20^0C overnight which resulted in the VATPase C gene cloned into the destination vector. Similarly, the C gene cloned in destination vector was

transformed into DH5α competent bacterial cells following the same protocol described above for the transformation of entry clone. Also, after transformation culturing of the transformed bacterial colonies was done similar to the entry clone. Following culturing of the transformed bacterial cells, the plasmid was extracted using plasmid mini prep kit (uses resuspension, lysis and neutralization solutions).

To check the presence of the VATPase C gene in the pMDC32 restriction enzyme EcoRI was selected and the banding pattern was determined with Vector NTI software. The banding pattern expected from this restriction enzyme was 9761bp, 1146bp and 345bp. The volume of the components and the incubation condition is presented below.

Components	Volume/reaction
EcoR I (promega)	0.2 µL
Promega EcoR I buffer (H)	1.5 µL
MilliQ water	11.3 µL
	13 µL
DNA	2 µL
	15 µL

The above solution was incubated overnight at 37^0C. Then, the digested DNA was run on agarose gel and the correct banding pattern was observed in the first clone which is shown in the first lane of figure below (Fig. 6)

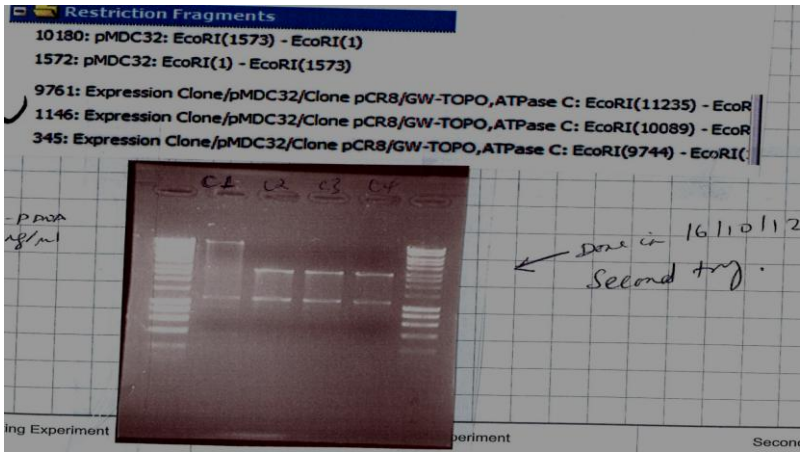


Figure 6. The restrictions digest of the pMDC32 to check the presence of VATPase C gene.

To further confirm that this clone indeed contain the VATPase gene, another restriction enzyme (Pvu I) was used. Similarly, the banding pattern to be generated by using this restriction enzyme was determined using Vector NTI software. It was 6671bp, 2719bp, 1675bp and 687bp. Consistent to the above result, the same clone showed the expected banding pattern confirming that it contains the VATPase C gene in the pMDC32 (Fig. 7)

All the below clones are derived from C7 clone in pMDC32 (destination vector)

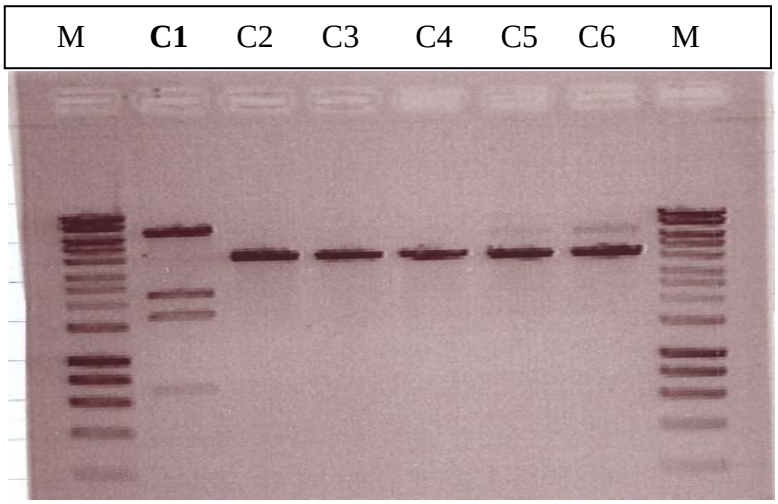


Figure 7. The Pvu I restriction enzyme digestion to further confirm the presence of VATPase C gene in pMDC32.

Finally, this clone that contains our gene was sent to the transformation lab for barley transformation.

Repeating the cloning of VATPase subunit D gene

Because the sequencing result for VATPase D gene had SNPs, the process of cloning has been done again following the steps described above. The gel picture showed the successful amplification of the gene using the same protocol described above. All the three lanes in the figure shows the same gene amplified in three tubes with similar condition (Fig. 8)

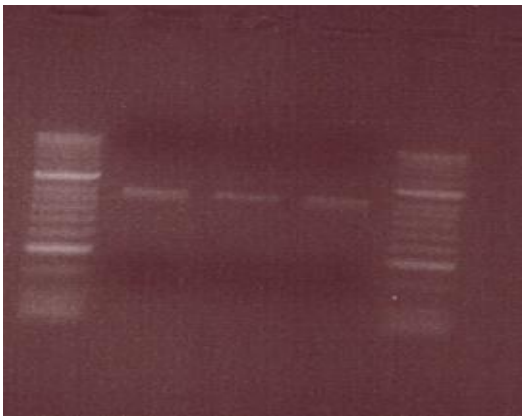


Figure 8. VATPase subunit D gene amplified using elongase enzyme

The gene was cloned into pCR8 entry clone and digested for correct orientation similar to the previous condition and also with similar enzymes. The double digestion was made using HpaI and XhoI and the expected band sizes were 1925bp, 1241bp, and 437bp. The D11 & D12 clones showed the correct orientation (Fig. 9). One of the clones which showed the correct orientation will be cloned into pMDC32.

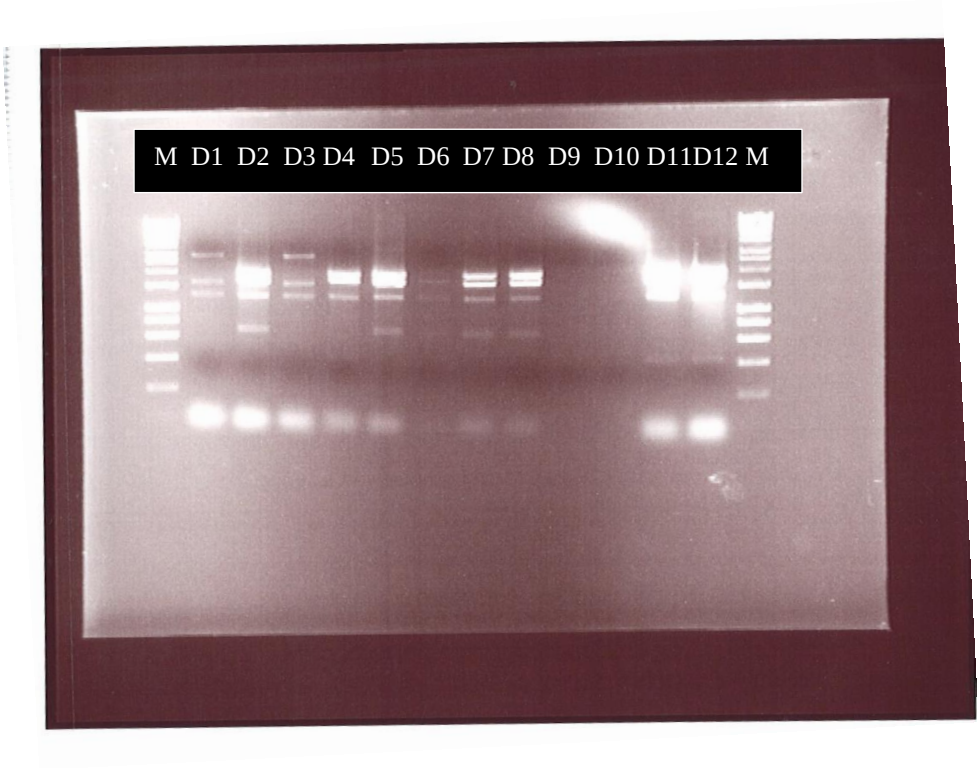


Figure 9. Double digestion to check correct orientation of V-ATPase D inserts.

Peptide sample preparation and analysis by nano liquid chromatography/ Tandem mass spectrometry

Peptide samples were generated using co-precipitation of proteins with trypsin as previously described (Wilson et al., 2010). Briefly, 100 µl aliquots of crude protein extract were clarified by centrifugation at 13,000 rpm and the supernatant precipitated with 9 volumes of ice-cold ethanol (2 hrs at -20). The protein pellets were resuspended in 90 µl denaturing buffer (7M urea, 2M thiourea in 30mM Tris pH 8.0) and subjected to sequential reduction, alkylation and trypsin digestion.

Peptide samples were analysed by nanoLC-MS/MS using an LTQ-Orbitrap XL (ThermoFisher Scientific, Waltham, MA, USA). Aliquots of tryptic peptides were loaded at 0.05 ml/min onto a C18 capillary trapping column (Peptide CapTrap, Michrom BioResources, Auburn, CA, USA) controlled by an Alliance 2690 Separations Module (Waters). Peptides

were then separated using a Surveyor MS Pump plus (ThermoFisher Scientific) on an analytical nanoHPLC column packed with 5 micron C18 ProteoPep II media (PicoFrit Column, 15 µm i.d. pulled tip, 10 cm, New Objective) as previously described (Al-Naseri *et al.* 2013).

The LTQ-Orbitrap XL was controlled using Xcalibur 2.0 software (ThermoFisher Scientific) and operated in data-dependent acquisition mode where survey scans were acquired in the Orbitrap using a resolving power of 60,000 (at 400 m/z). MS/MS spectra were concurrently acquired in the LTQ mass analyser on the eight most intense ions from the FT survey scan. Charge state filtering, where unassigned and singly-charged precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count 1, repeat duration 30 s, exclusion list size 500) were used. Fragmentation conditions in the LTQ were: 35% normalised collision energy, activation q of 0.25, 30 ms activation time and minimum ion selection intensity of 500 counts.

Database searching and criteria for protein identification

Centroid mode spectra acquired were converted from .RAW files into .mzXML peak list files using the msConvert command (Proteowizard). The extracted MS/MS data were searched against the Barley protein database of 36,116 entries downloaded from the National Centre for Biotechnology Information on 21/05/2013, to which sequence for the Arabidopsis sodium hydrogen exchange protein -1 had been appended (>sp|Q68KI4|NHX1_ARATH Sodium/hydrogen exchanger 1 OS=Arabidopsis thaliana GN=NHX1). Semi-tryptic searches using parent ion and fragment ion mass tolerances of 10 ppm and 0.5 Da, respectively, were performed using X!Tandem running in the Computational Proteomics Analysis System (CPAS), an open-source bioinformatics resource for analysing large proteomics datasets (Rauch *et al.* 2006).

S-carboxamidomethylation of cysteine residues was specified as a fixed modification and oxidation of methionine was specified as a variable modification. The Peptide Prophet and Protein Prophet algorithms were applied to the X!Tandem search results to assign probabilities to peptide and protein matches, respectively (Keller *et al.* 2002; Nesvizhskii *et al.* 2003). Peptide-spectrum matches were accepted if the peptide was assigned a probability > 0.95 by the Peptide Prophet algorithm. Protein identifications were accepted if the protein contained two or more unique peptide sequences and the protein was assigned a probability > 0.99 by the Protein Prophet algorithm.

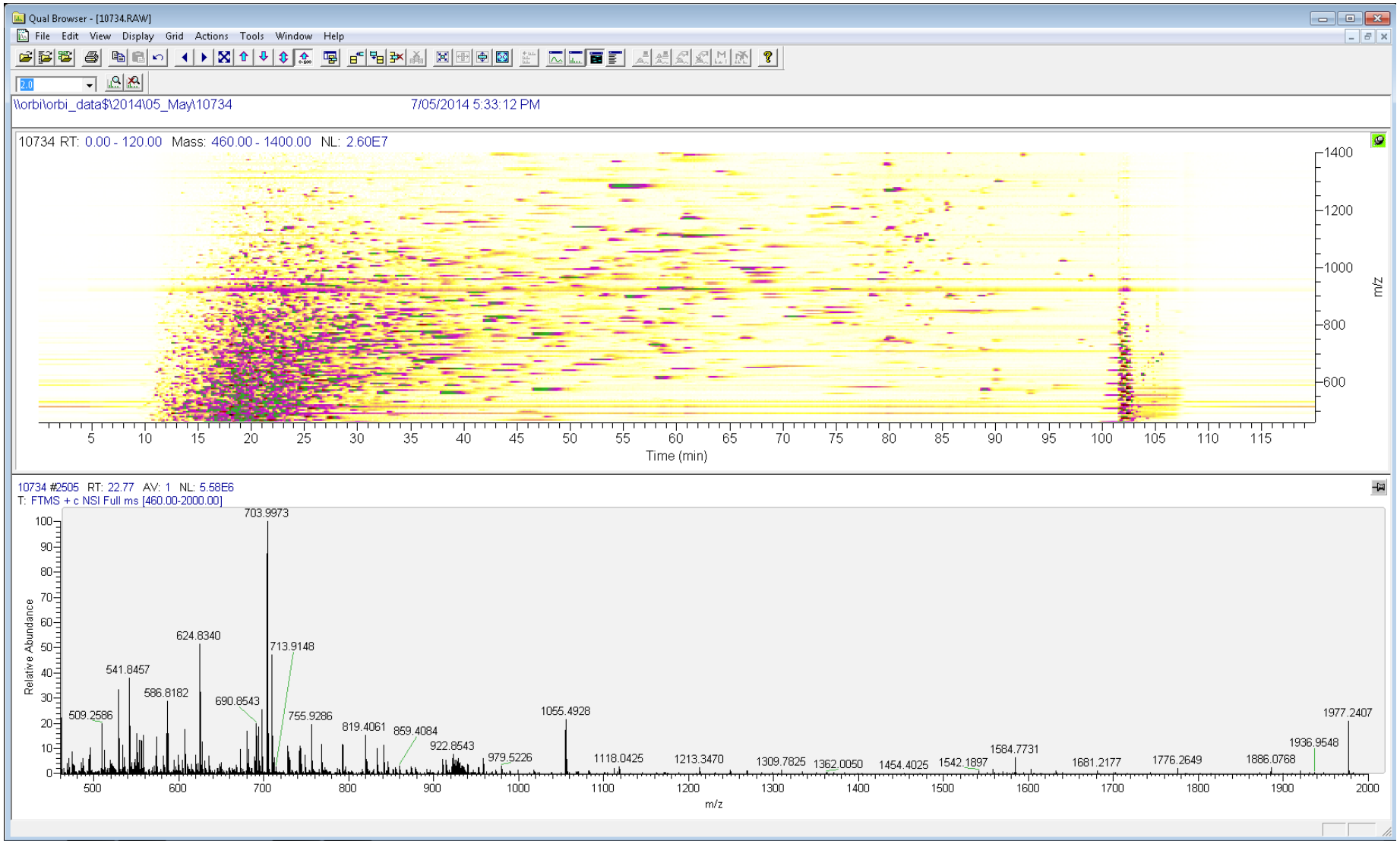
Al-Naseri, A, Bowman, JP, Wilson, R, Nilsson, RE, & Britz, ML 2013,′ Impact of Lactose Starvation on the Physiology of *Lactobacillus casei* GCRL163 in the Presence or Absence of Tween 80′, *Journal of Proteome Research*, Vol. 12, no. 11, pp 5313-5322.

Rauch, A, Bellew, M, Eng, J, Fitzgibbon, M, Holzman, T, Hussey, P, Igra, M, Maclean, B, Lin, WC, Detter, A, Fang, R, Faca, V, Gafken, P, Zhang, H, Whitaker, J, States, D, Hanash, S, Paulovich, A, & Macintosh, MW 2006, ′Computational Proteomics Analysis System (CPAS): An Extensible, Open-Source Analytic System for Evaluating and Publishing Proteomic Data and High Throughput Biological Experiments′, *Journal of Proteome Research*, vol. 5, no.1, pp. 112-121.

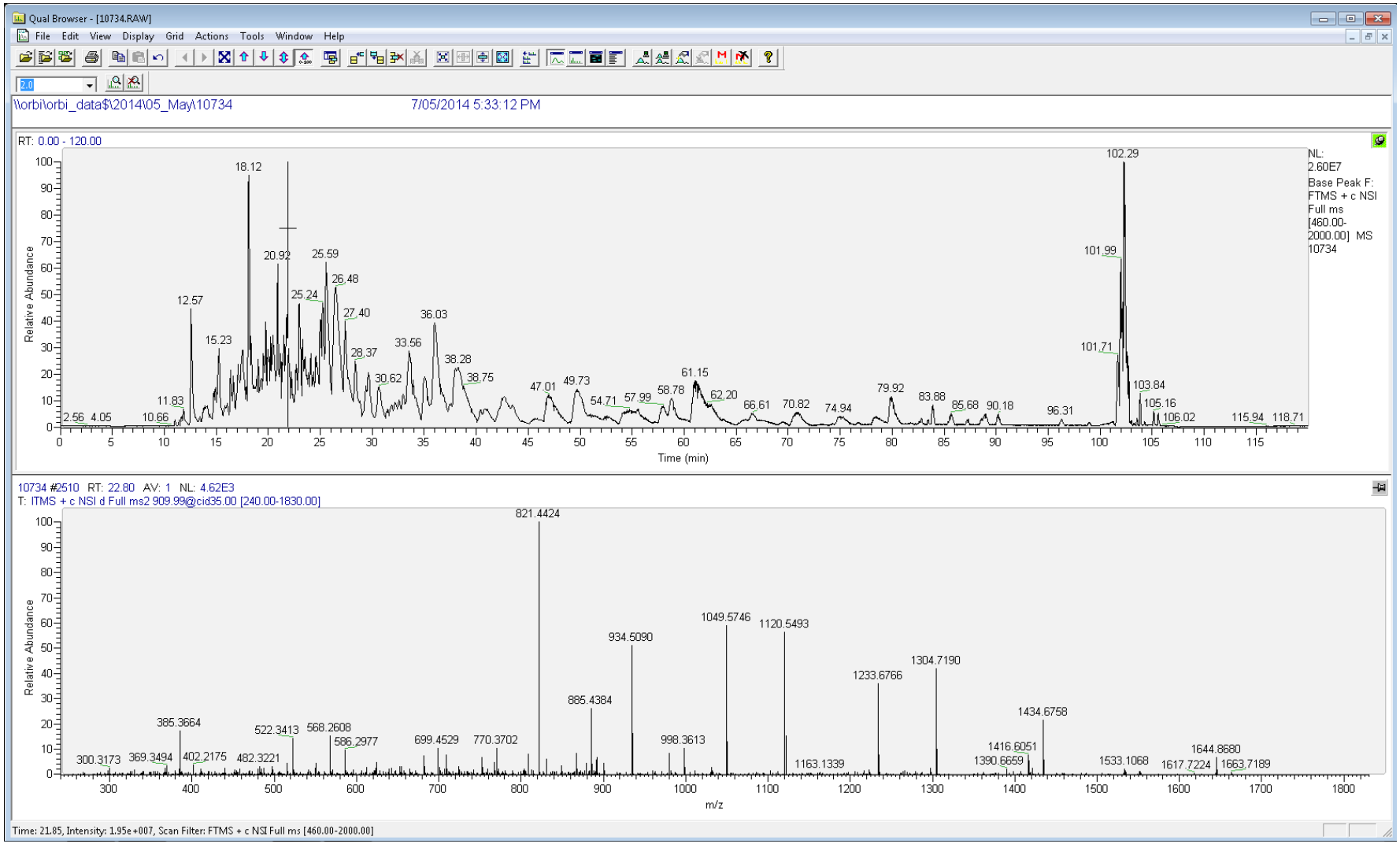
Keller, A, Nesvizhskii, AI, Kolker, E, & Aebersold, R 2002, ′Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search′, Analytical Chemistry, vol.74, pp. 5383-92.

Nesvizhskii, AI, Keller, A, Kolker, E, & Aebersold, R 2003, ′A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry′, Analytical Chemistry, vol. 75, pp. 4646-58.

Wilson, R, Diseberg, AF, Goordon, L, Zivkovic,S, Tatarczuch, L, Mackie, EJ, Gorman, JJ & Bateman, JF 2010,′ Comprehensive profiling of cartiliage extracellular matrix formation & maturation using sequential extraction & label-free quantiative proteomics′, Molecular & Cellular Proteomics, vol. 9, isssue 6, pp. 1296-1313.



1. Sample ID #10734 ion map view (intensity scaled by colour – green is most intense signal)
2. Example high-resolution FTMS survey scan (resolving power 60,000)



3. Sample ID #10734 base peak chromatogram
4. Example MS/MS spectrum for precursor ion m/z 909.99 – see details on following slides

gi|112684|sp|P29305.1|1433A_HORVU (1433A_HORVU)

Sequence Mass: 29,352
AA Coverage: 56% (146 / 262)
Mass Coverage: 55% (16,139 / 29,352)

MSTAEATREE NVYMAKLAEQ AERYEEMVEF MEKVAKTADV GELTVEERNL LSVAYKNVIG ARRASWRIIS SIEQKEESRG NEAYVASIKE YRTRIETELS KICDGILKLL DSHLVPSATA AESKVFYLKM KGDYHRYLAE FKAGAERKEA AENTLVAYKS AQDIALADLP TTHPIRLGLA LNFSVFYYEI LNSPDRACNL AKQAFDEAIA ELDSLGEESY KDSTLIMQLL RDNLTLWTSD NAEEGGDEIK EAASKPEGEG HS

Annotations for 1433A_HORVU

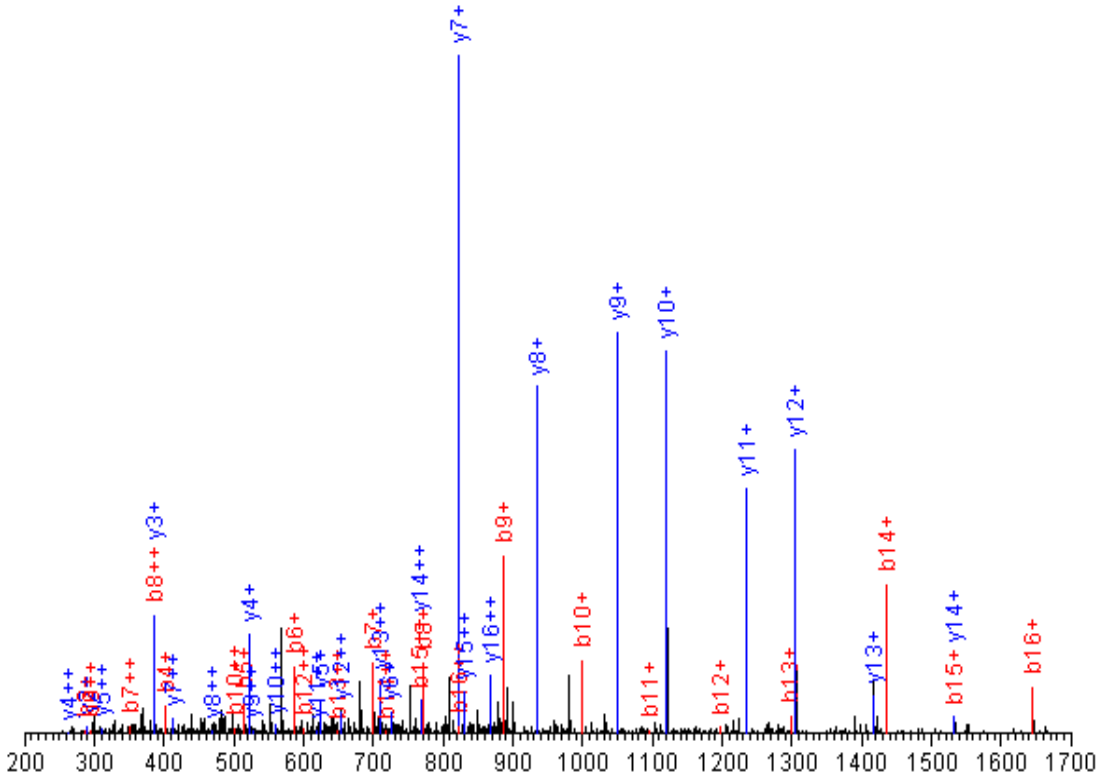
Sequence name: 1433A_HORVU
Description: 14-3-3-like protein A - Hordeum vulgare (Barley)
Gene Name(s):
Organisms(s): Hordeum vulgare

Genbank IDs	GIs	Swiss-Prot Accessions	Swiss-Prot Names	Ensembl	IPI numbers	GO Categories
	112684	P29305.1 P29305	1433A_HORVU			

Peptides

Peptide Filter: (PeptideProphet >= 0.95)
Peptide Sort: Protein ASC, Peptide ASC

Scan	Z	Hyper	Next	B	Y	Expect	Ion%	CalcMH+	dMass	PepProphet	Peptide	SeqHits
4379	2+	46.8	34.2	10.5	11.5	0.5300	83%	1189.6609	+0.0050	0.9937	K.DSTLIMQLLR.D	27
4454	2+	46.9	37.7	10.7	11.5	0.2700	83%	1189.6609	+0.0050	0.9934	K.DSTLIMQLLR.D	27
1490	2+	47.6	45.2	7.4	10.2	0.0360	80%	1208.6157	+0.0040	0.9946	K.EAAENTLVAYK.S	3
1900	2+	72.3	22.9	11.9	13.0	0.0000	70%	1638.8697	+0.0040	1.0000	K.LLDSHLVPSATAAESK.V	3
10325	3+	46.7	34.2	11.6	11.0	0.0056	17%	3268.5930	+0.0170	0.9983	K.Q^AFDEAIAELDSLGEESYKDSTLIMQLLR.D	3
5311	2+	67.7	34.5	9.6	12.2	0.0000	56%	2114.9764	+0.0090	1.0000	K.QAFDEAIAELDSLGEESYK.D	3
5350	3+	49.1	45.5	10.0	10.5	0.3600	29%	2114.9760	+0.0150	0.9753	K.QAFDEAIAELDSLGEESYK.D	3
5401	2+	49.8	27.4	8.8	11.8	0.0007	44%	2114.9764	-0.0010	0.9997	K.QAFDEAIAELDSLGEESYK.D	3
2510	2+	79.2	35.1	10.8	13.5	0.0000	72%	1818.9708	+0.0050	1.0000	K.SAQDIALADLPTTHPIR.L	3
2514	3+	61.1	37.1	10.0	12.4	0.0013	38%	1818.9708	+0.0040	1.0000	K.SAQDIALADLPTTHPIR.L	3
1667	2+	45.9	40.0	8.7	10.9	0.0500	68%	1318.6485	+0.0070	0.9967	K.TADVGELTVEER.N	3
2836	3+	107.9	46.9	7.6	11.1	0.0000	32%	3199.4298	+0.0050	1.0000	R.DNLTLWTSDNAEEGGDEIKEAASKPEGEGH.S	3
1019	2+	73.9	35.8	8.8	12.3	0.0000	100%	1418.7485	+0.0050	1.0000	R.IISSIEQKEESR.G	12
860	2+	41.6	30.2	11.4	9.8	0.2400	88%	1076.5946	+0.0030	0.9654	R.TRIETELSK.I	3
2518	2+	57.3	28.6	10.1	12.2	0.0002	100%	1334.5643	+0.0060	1.0000	R.YEEMVEFMEK.V	12



b^{2+}	b^{+}	#	AA	#	y^{+}	y^{2+}
44.5470	88.0861	1	S	17		
80.0864	159.1649	2	A	16	1732.9774	866.9927
144.1518	287.2957	3	Q	15	1661.8986	831.4533
201.6961	402.3843	4	D	14	1533.7679	767.3879
258.2758	515.5437	5	I	13	1418.6793	709.8436
293.8152	586.6225	6	A	12	1305.5198	653.2639
350.3949	699.7819	7	L	11	1234.4410	617.7245
385.9343	770.8607	8	A	10	1121.2816	561.1448
443.4786	885.9493	9	D	9	1050.2028	525.6054
500.0584	999.1088	10	L	8	935.1142	468.0611
548.6167	1096.2255	11	P	7	821.9547	411.4813
599.1692	1197.3305	12	T	6	724.8381	362.9230
649.7218	1298.4356	13	T	5	623.7330	312.3705
718.2923	1435.5767	14	H	4	522.6279	261.8179
766.8507	1532.6934	15	P	3	385.4868	193.2474
823.4304	1645.8528	16	I	2	288.3701	144.6890
		17	R	1	175.2107	88.1093

MS/MS spectrum (daughter ions of peptide mz 909)